CLOSURE OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION CHAMBER ON LAB-ON-CHIP USING THERMAL-RESPONSIVE VALVE

by

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ABSTRACT

Point-of-care tests for nucleic acids are important for the diagnosis and management of infectious and genetic diseases, biowarfare agents, and for drug research. Recent integration of loop-mediated isothermal amplification (LAMP) onto a lab-on-chip (LOC) platform allows sensitive and specific, detection of target DNA or RNA sequences for point-of-care (POC) applications. However, because of the high risk of contamination to LAMP products, robust and simple methods of hermetically sealing the reaction chamber are essential. In addition, having a method for isolation of nucleic acids at the level of the chip is more effective for POC applications because a laboratory setting is not required to complete the analysis.

This report describes an integrated, polymer-based cassette which was designed for detection of DNA/RNA target-sequences by using LAMP and a solid-state nucleic acid purification membrane. The LAMP chamber seals using a self-actuated thermal-responsive valve made from expandable microspheres suspended in Polydimethylsiloxane (PDMS). A flow-through, Flinders Technology Associates membrane (Whatman FTA®) was installed on the cassette with the expectation of providing isolation and purification of nucleic acids. The cassette was designed, fabricated and the efficacy of the valve to seal the LAMP chamber was investigated. The valve underwent expansion and held pressures of 233 +/− 5.0 kPa without signs of leakage. A portable, battery-powered, polyimide-based thin film heater, placed outside the cassette, provided thermal control. In addition, a LAMP primer set was designed for

the serotonin receptor 5HTR1A promoter gene using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html) for LAMP detection of neurotransmitter serotonin. The integrated, portable LAMP cassette will be attractive in global health-care challenges, mainly in resource-poor locations, where the availability of laboratory equipment and/or trained personal is restricted.

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INTRODUCTION

POC tests can provide immediate results for surveillance of epidemics (Donovan et al. 2004), early detection of biomarkers in emergency medicine (Caragher et al. 2002) , and may be adapted for detection of biowarfare agents (Louie et al. 2009). POC tests can potentially eliminate the dependence on large centralized laboratories and hospitals, allowing "sample-to-answer" personalized diagnostics at the bedside, in the doctor's office, or at home. Perhaps the most beneficial usage of POC tests are for resource limited locations, where availability of laboratory equipment and trained personal are limited. There is also great interest in using nucleic acid amplification techniques for some types of POC testing (Mori et al. 2009).

Nucleic acid amplification is a widely demonstrated resource for molecular diagnostics. In particular, the polymerase chain reaction (PCR) and real-time PCR, are conventional methods for DNA/RNA amplification. However, they generally require the use of a thermocycler, which can be time-consuming and expensive. Currently, a method known as loop-mediated isothermal amplification, or LAMP, provides amplification with great specificity, eliminating the requirement for a thermocycler (Notomi et al. 2000). Complete reaction times are much shorter because there is no time loss due to thermal changes. Additionally, a constant enzymatic temperature (i.e. isothermal condition) is maintained, allowing high concentrations of amplified product to be used.

The need for rapid and inexpensive POC tests has led to the integration of nucleic acid amplification onto lab-on-chip (LOC) platforms (Gervais et al. 2011), (Olasagasti et al.

2012) . A wide variety of laboratory functions can be integrated onto these miniaturized platforms, significantly reducing analysis time. Often, these platforms can be operated without the need for trained personnel.

Recently, much effort has been devoted to integrating LAMP onto LOC platforms (Fang, Kong et al. 2010). Although robust, very few groups have reported both LAMP, and purification/isolation of nucleic acids at the level of the chip. On-chip nucleic acid isolation may be more suited for the POC because target-samples are added without additional steps or laboratory equipment. In addition, the large majority of platforms that exercise nucleic amplification do not use valve systems. Using valves to seal the reaction chamber during amplification greatly reduces contamination (Whelan 2001).

Samel et al reported on self-actuating thermally responsive valves for microfluidic applications using a mixture of on PDMS and expandable microspheres (Samel et al. 2007) . These valves are easy to fabricate and inexpensive. The thermoplastic valves have been used on several LOC platforms (Roxhed et al. 2006), (Samel et al. 2007), (Liu et al. 2011).

The purpose of this study was to develop an inexpensive, portable POC test for detection of DNA/RNA sequences using a LAMP chamber. A single thermally-responsive, self-actuating valve, fabricated from a composite of PDMS and expandable microspheres, seals the chamber. The platform is expected to provide on-chip nucleic acid purification by using a flow through, Flinders Technology Associates membrane (Whatman FTA®). The membrane also helps to remove air bubbles prior to amplification. This report

describes the design and construction of the cassette and characterizes the efficacy of the valve. Upon heating, the valve expanded isolating the amplification chamber. The valve can be heated using a portable polyimide-based thin film heater that can be battery powered and placed external to the cassette. Real-time detection of LAMP products is proposed using a compact fluorescence reader.

BACKGROUND AND LITERATURE REIVEW

Nucleic acid amplification

Introduction to PCR

Since the invention of the Polymerase Chain Reaction in the mid- 80's by K. Mullis et al., PCR is now a common and invaluable tool used in genetics, biology, and medicine. (Shampo and Kyle 2002). Current PCR applications includes classification of organisms (Hotzel et al. 1996) , genotyping (Taberlet et al. 1996), cancer research (Mocellin et al. 2003), DNA fingerprinting (McClelland et al. 1994), drug discovery (Covault et al. 2003) , and notably the detection of genetic or infectious diseases (i.e. bacterial and viral) (Gasparini et al. 1993), (Josephson et al. 1993), (Nairn et al. 2003).

PCR is a powerful *in-vitro* enzymatic technique that enables exponential amplification of DNA sequences (Saiki et al. 1988). A specific region of a DNA, known as the target sequence is amplified. These target sequences are on the order of 10-40 kilo-base pairs (kb) long (Cheng et al. 1994). A kilobase (kb) is equal to 1000 base-pairs of DNA (or RNA) comprised of hydrogen bonded nitrogenous bases, with each base originating from the opposite complementary DNA strand. The end result allows a single copy or several copies of a short DNA sequence to be amplified, (i.e. replicated) up to a million copies within a few hours.

The majority of PCR methods rely on thermal cycling, which consists of alternative cycles of heating and cooling in a defined series of temperature stages. The process of thermal-cycling results in consecutive DNA melting and enzymatic replication. During

thermal cycling, the first stage uses high temperatures to denature or physically separate the two strands of DNA. This stage is called DNA melting. The second stage uses a lower temperature allowing a set of oligonucleotides primers to selectivity anneal to the target DNA sequence and then bind to the portion of DNA to be replicated. Once the template is laid, the temperature is elevated, and each strand is selectively replicated by DNA polymerase through the addition of deoxynucleotide triphosphates (dNTPs).

In summary the cycle consists of the following:

1. Denaturation of DNA via DNA melting at high temperatures to convert double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA).
2. Annealing or hybridization of two oligonucleotides primers that selectively amplify the target DNA sequence.
3. Extension of the target DNA sequence via DNA polymerase.

Following each extension, the copies produced are known as amplicons. When the process repeats, the amplicons are "re-amplified," reusing the same primers. Figure 1.1 shows a summary of the steps.

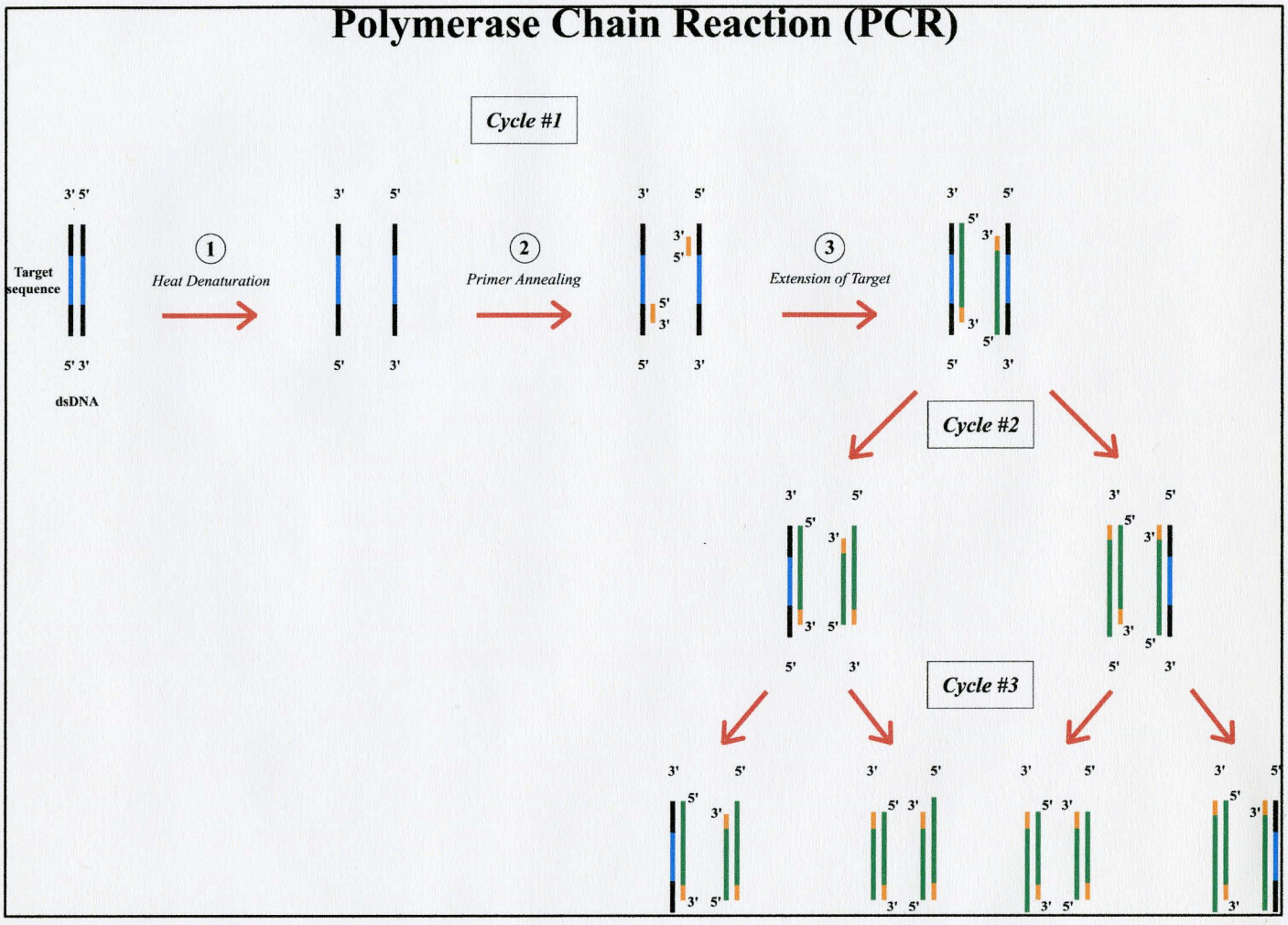

Figure 1.1 – PCR amplification consists of 3 steps in which each cycle results in doubling of the target-sequence. Step 1 consists of denaturation of the dsDNA from heating at elevated temperatures. Primers then anneal to the target-sequence to be amplified. Step 3 involves primer extension of the target-sequence via DNA polymerase.

In order to understand the fundamental differences between PCR methods, it is important to gain insight into the kinetics of the process. PCR is modeled using three phases: the exponential, linear, and terminal phase (Gevertz et al. 2005). During the exponential

phase, the quantity of initial DNA is doubled precisely with every other cycle. The linear phase occurs when reaction components are being consumed, and the formation of products starts to degrade. The plateau (terminal) phase occurs when the generation of PCR products has stopped because lack of critical reactant components (Figure 1.2).

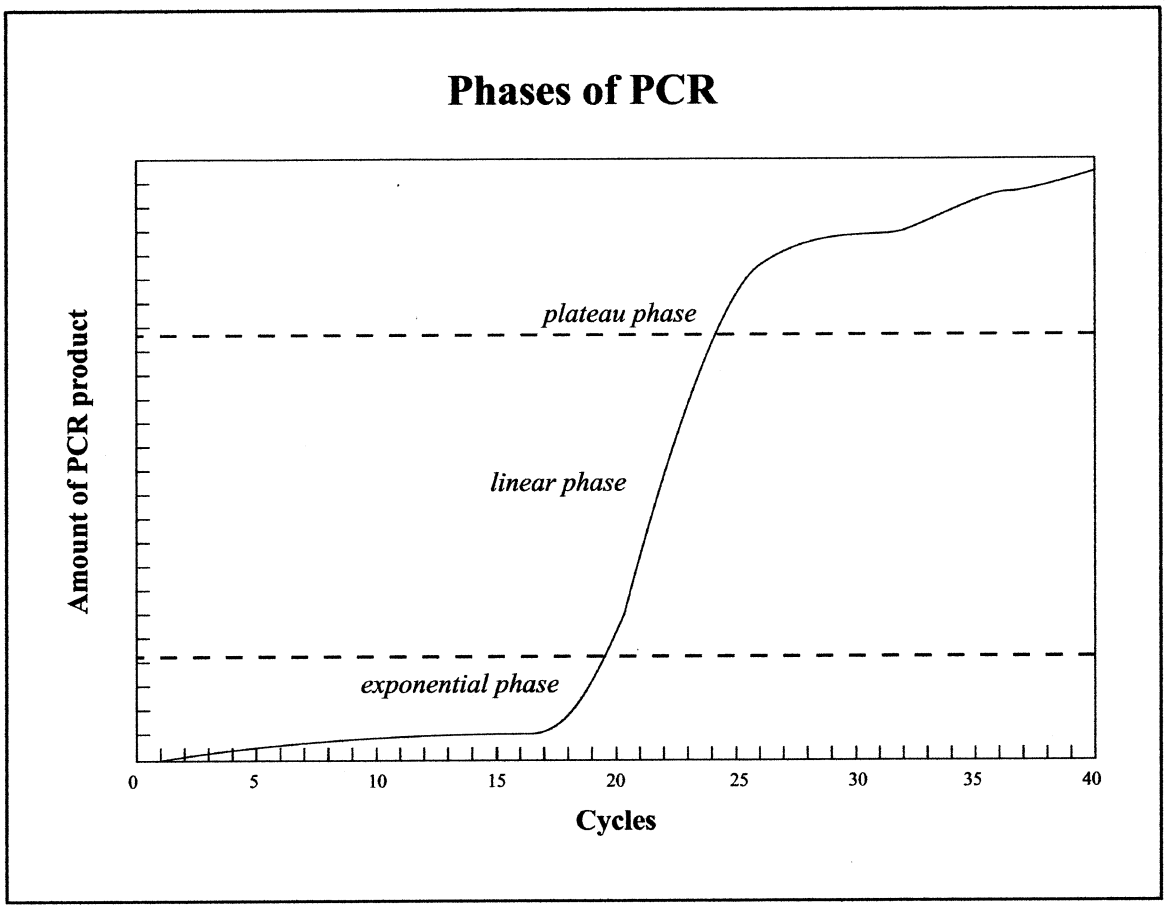

Figure 1.2 –Kinetic phases of PCR. PCR is separated into three distinct phases. During the exponential phase, amplicons are doubled with every other cycle. Linear phase indicates the slowing of the reaction in which reagent components are being consumed and products formation begins to degrade. The reaction stops during the plateau phase.

For conventional PCR , following amplification, gel electrophoresis is often used to analyze products (Kochanowski et al. 1999) . Gel-electrophoresis is a method that uses electrical current to separate proteins or DNA based on charge and/or size (Kryndushkin et al. 2003). This can make conventional PCR time consuming because the PRC products must be loaded into a gel-electrophoresis device after the PCR reaction. This type of post-PCR analysis is often referred to as *end-point detection*, in which PCR products are quantified at the plateau phase, after a reaction has ceased. In end-point detection, samples that start with the same quantity of PCR components at the beginning of the reaction end up at different points in the plateau phase, due to the different kinetics each sample had during cycling (Schmittgen et al. 2000) .

During exponential amplification, a theoretical doubling of amplicons occurs with each cycle, yielding a consistent relationship between initial and amplified DNA (Jung et al. 2000). Therefore, it is more efficient to take measurements during the exponential phase of amplification.

RT-PCR

Real time PCR, otherwise known as quantitative PCR (qPCR) (VanGuilder et al. 2008), measures amplicons during the exponential phase. Therefore, you are able to detect and quantify products that are generated during each cycle proportional to the amount of the initial DNA before the start of the PCR process (Heid et al. 1996). This is accomplished using additional chemistry (reporters) to detect the products formed from each cycle.

Two methods of reporter-probe chemistries are commonly used in qPCR. These include

non-specific fluorescent dyes that intercalate into double stranded DNA (dsDNA), and sequence-specific probes consisting of oligonucleotides labeled with fluorescent reporters that permit detection upon hybridization of the reporter to the complementary DNA strand (Whitcombe et al. 1999). A thermocycler adapted to record the results during each amplification cycle is generally used. This method enables both quantification and detection of amplicons during "real-time" as opposed to conventional PCR. Average reaction times can range from 1-2 hours when a standard thermocycler is used.

A RNA strand can be amplified using qPCR. Amplifying a RNA strand is accomplished first by reverse transcription of the RNA target-sequence into DNA using the enzyme reverse transcriptase (Freeman et al. 1999).

Loop-mediated isothermal amplification

Conventional PCR and RT-PCR amplification requires a thermal cycling process. The addition of a thermal-cycler can be expensive, complex, and time consuming. Loop-mediated isothermal amplification, or LAMP, is a single-step, isothermal nucleic acid amplification technique (Notomi et al. 2000). LAMP takes place at a constant temperature of approximately 60–65 degrees Celsius, in which the target-sequence is amplified using a set of 4 primers to detect 6 regions on the target-genome. This is accomplished though single-stand displacement reactions coupled to replication activity. A single strand displacement reaction is the process in which two complementary stands hybridize together, displacing one or more previous hybridized strands. This occurs because of the energetically favorable formation of new base pairs, and entropies gained

during disassembly (Mills et al. 1999) . Consequently, this allows for isothermal operation. LAMP may also be combined with a reverse transcriptase to allow the detection of RNA (Parida et al. 2005).

Amplification occurs in a single step, where a mixture of reagents, target-sample, replicating polymerase, and reporters are placed into a single test-tube. In 15-60 minutes, 100-1000 times DNA amplification has been reported with high replication efficacy (Tomita et al. 2008) . The main advantage to this method is, because 4 primers are used to detect 6 regions of target-genome, very high specificity is common, in which the presence of amplified product is indicative of presence of the target gene. For example, Higashimoto, et al demonstrated discrimination of a single nucleotide difference in Varicella-Zoster virus vaccine and wild-type strains using the LAMP method (Higashimoto al. 2008) . Additionally, LAMP generally produces higher DNA amplification than real-time and conventional PCR methods (Chen et al. 2011) .For example, Parida et al reported a greater sensitivity of the LAMP compared to RT-PCR. They concluded that the LAMP assay had a 10-fold greater sensitivity with a positive result within 17 minutes for strains of the West-Nile virus (Parida et al. 2004). A variety of pathogens and infectious diseases, have successfully been detected using LAMP, including bacterial (Yokoyama et al. 2010), fungal (Ohori et al. 2006) and viral target-sequences (Wei et al. 2012).

There are several disadvantages to the LAMP method. First, LAMP requires a complicated design of multiple primers. Second, the final product is a complex mixture

of stem–loop cauliflower-like DNA, consisting of alternately inverted repeats of the target-sequence of the same strand (Soliman et al. 2005). Third, detection using gel-electrophoresis is difficult. On the gel, LAMP products are seen in a ladder-like pattern as compared to the single strand segments seen in PCR. Lastly, there are extra steps required to obtain uniform, single-stranded DNA. These extra steps involve use of a thermostable TspRI restriction enzyme to digest the amplification product, and an additional primer to displace the single-stranded DNA by primer extension.

Table 1 provides a list of the advantages and disadvantages for the LAMP method.

Advantages	**Disadvantages**
In general, offers greater specificity because 4 primers are used to recognize 6 distinct regions of the target-sequence.	Requires a complicated design of multiple primers.
Whole amplification reaction takes place continuously under isothermal conditions at 60-65 °C.	The final product is a complex mixture of stem–loop cauliflower-like DNA structures of various sizes.
Does not require a thermocycling machine to separate the two DNA strands and then amplify the required fragment.	Detection using gel-electrophoresis is difficult. LAMP products are seen in a ladder-like pattern as compared to the single strand segments seen in PCR
Total cost can be reduced, as LAMP does not require sophisticated equipment to provide thermal control.	There are extra steps required to obtain uniform, single-stranded DNA from LAMP product.

Table 1.1 – Advantages and disadvantages of LAMP.

LAMP reporters

Several reporter chemistries are used in LAMP. These include the addition of intercalating non-specific fluorescent dyes, e.g. SYBR Green and SYTO series (Iwamoto et al. 2003), (Njiru et al. 2008) , magnesium-ions (Mori et al. 2001), or magnesium-loaded calcein in solution (Tomita et al. 2008). The latter two dyes take advantage of the by-product formation of magnesium pyrophosphate produced during DNA synthesis. Real-time detection of amplified products, is often observed through an increase of the turbidly (colorimetric detection) from magnesium pyrophosphate or fluorescent signals from the intercalating dyes or magnesium-loaded calcein ions.

The ability of the LAMP reaction to produce large amounts of DNA results in large amounts of by-products. These by-products yield a white precipitate known as magnesium pyrophosphate. Magnesium pyrophosphate is formed as a result of DNA polymerization by DNA polymerase. This occurs during replication when pyrophosphate ions are released from dNTPs, and magnesium ions, added as a reagent, react with pyrophosphate ions yielding the precipitate. A positive test is indicated by an increase in turbidity (Mori et al. 2001). However, the visual signal is often weak, and may be subjected to observer bias. Therefore, methods for better recognition are desired.

Pyrophosphate ions bind with high affinity to various metallic ions to form insoluble salts (Mori, Nagamine et al. 2001). Calcein is a metal indicator, which yields a fluorescent signal by forming a complex with a divalent metallic ion such as calcium and magnesium (Hoelzlwallach 1964), (Demertzis 1988). In LAMP, manganese ions are used, coupled

with calcein as the fluorescent metal indicator (Tomita et al, 2008), which quenches the reaction (i.e. fluorescent signal). A calcein/manganese reagent is added before the reaction proceeds. During DNA synthesis, calcein replaces manganese ions with newly formed pyrophosphate ions, forming the calcein-manganese complexes. Under general UV light, a green emission spectrum is produced and increases if the target DNA is present in solution. However, the signal produced by the calcein/manganese method is lower and less sensitive (Goto et al. 2009) than that of SYBR dye method. Furthermore, the requirement for a UV-light source can be expensive and hazardous.

SYBR® and SYTO green dyes are reported to have a large increase in fluorescent intensity upon interaction with DNA. They are robust and commonly used fluorescent nucleic acid dyes (Matsui et al. 2004) and may be used in both UV and visible light. The fluorescence intensity is proportionate to the amount of amplified products produced. During each stage of amplification, you may monitor the products. However, because they are non-specific, any free DNA may become bound generating false positive results. These mismatches are usually caused by primer-dimer or non-specific binding, and are evaluated using a melting-curve analysis where homogeneity is determined from conformation of single thermal-transitions curves (Zipper et al. 2004) .

There are several advantages to using fluorescent dyes. The signal produced from intercalating dyes is often stronger than that of magnesium-calcein reagent. Furthermore, a more dramatic change is seen compared to the calcein/manganese method (Goto et al. 2009). In general, the color of the green dye changes from an orange to a bright green

fluorescence when combined with DNA. This is generally accomplished by using a UV or blue light source. This may prove important for resource-limited locations where blue-light excitation can be easily established without the need of UV light.

The major drawback to this method stems from the fact that SYBR I green dye, if used directly as an initial reagent, will inhibit the LAMP reaction (Monis et al. 2005). Therefore, SYBR I green dye is generally added after the reaction has ceased. Notably, this creates a greater chance for contamination because of the large amounts of amplified products present in solution. Several groups have implemented different methods to avoid this by separating the SYBR green I dye from LAMP reagents until after the reaction is finished. Tao et al demonstrated an isolated LAMP vial that used microcrystalline wax-dye capsules filled with the dye (Tao et al. 2011) . These capsules delivered the dye following amplification through melting of the wax at temperatures of 95 degrees Celsius. However, this may add greater reagent costs, as well as imposing greater temperature requirements.

SYTO green dyes, specifically SYTO-9, SYTO-13, and SYTO-16, have no significant effect on PCR efficiency at high concentrations (Chen et al. 2011). SYTO 9 green dye can be added as an initial reagent. This may have several advantages. First, there is no need for a complex way of separating the dye until the reaction has been completed. Second, this enables real-time detection of the LAMP product. A corollary to this is, when only the presence of the target-analyte is desired, real-time detection may be able to give a positive result in much less time because amplification of a target-sequence can be

confirmed before the reaction is completed.

Visualization of LAMP products

Compared to conventional PCR, detection of LAMP products using gel-electrophoresis is only carried out in instances to check for false-positive results, or to see if amplification was indeed carried out by the LAMP primers. Furthermore, this method has a risk of contamination because of the large concentration of amplified products (Tao et al. 2011). As a result, LAMP is often carried out in closed reaction vials..

In general, the detection of the amplified LAMP products is determined by the "naked-eye" through the presence/change of turbidity or fluorescence signal inside a closed reaction vial. Semi-concentration/quantification of target-analyte is carried out by measuring the fluorescent or turbid signal as a function of time. Such a task generally requires additional equipment for "real-time" detection.

Real-time measurements of turbidity can be accomplished by the use of a photometer such as the Loopamp Real-Time Turbidimeter that controls the reaction temperature, time, and detects the white turbidity caused by magnesium pyrophosphate precipitates during specific time-intervals (Eiken, Teramecs). However, this system is expensive and restrictive requiring large operating voltages and space requirements.

The use of small mobile fluorescence detection system may be used to accomplish real-time LAMP (RT-LAMP) because only a small amount of sample/reagents are used

during reaction. For example, many portable optical devices use LED excitation as a light source coupled with a low noise Si-photodiode for fluorescence detection. Such detectors have been made for the point-of-care where a reduction of moving parts, low voltage requirements, and use of confocal optics, allow the detector to integrate into various applications (Fluo Sens Integrated, Qiagen). The detectors are generally connected to a computer through a USB interface. However, some systems are stand-alone. A plot of the fluorescence intensity as a function of time is then provided on a computer screen or LED display. Additional software provides real-time acquisition of the data.

LAMP primers

Four primers are used to detect 6 target-gene regions (Figure 1.4). These 6 regions are known as the F3, F2, and F1 located on the 3 prime side, and the B1, B2, and B3 located on the 5 prime side of a target DNA strand.

The 4 primers are denoted as the *Forward-inner primer* (FIP), *Forward Outer Primer* (F3), *Backward Inner Primer* (BIP), and the *Backward Outer Primer* (B3).

A description of each of the primers is as follows:

- FIP is comprised of the F2 sequence at the 3 prime ends and is complementary to the F2c region, and the same sequence as the F1c region at its 5 prime end.
- F3 primer consists of the F3 region that is complementary to the F3c region.
- BIP consists of the B2 region at the 3 prime end that is complementary to the B2c region, and the same sequence as the B1c region at the 5 prime end.

- B3 Primer consists of the B3 region that is complementary to the B3c region.

FIP initiates LAMP and is later replaced by the BIP. Loop primers can be used to increase the replication rate during LAMP (Nagamine et al. 2002). This is done by using additional primers that hybridize to regions that are not already hybridized from the inner primers (i.e. FIP and BIP) via a different reactive mechanism. Loop primers can achieve reaction times that are half that of the original LAMP method. The total time of detection using loop-primers is around 1 hour.

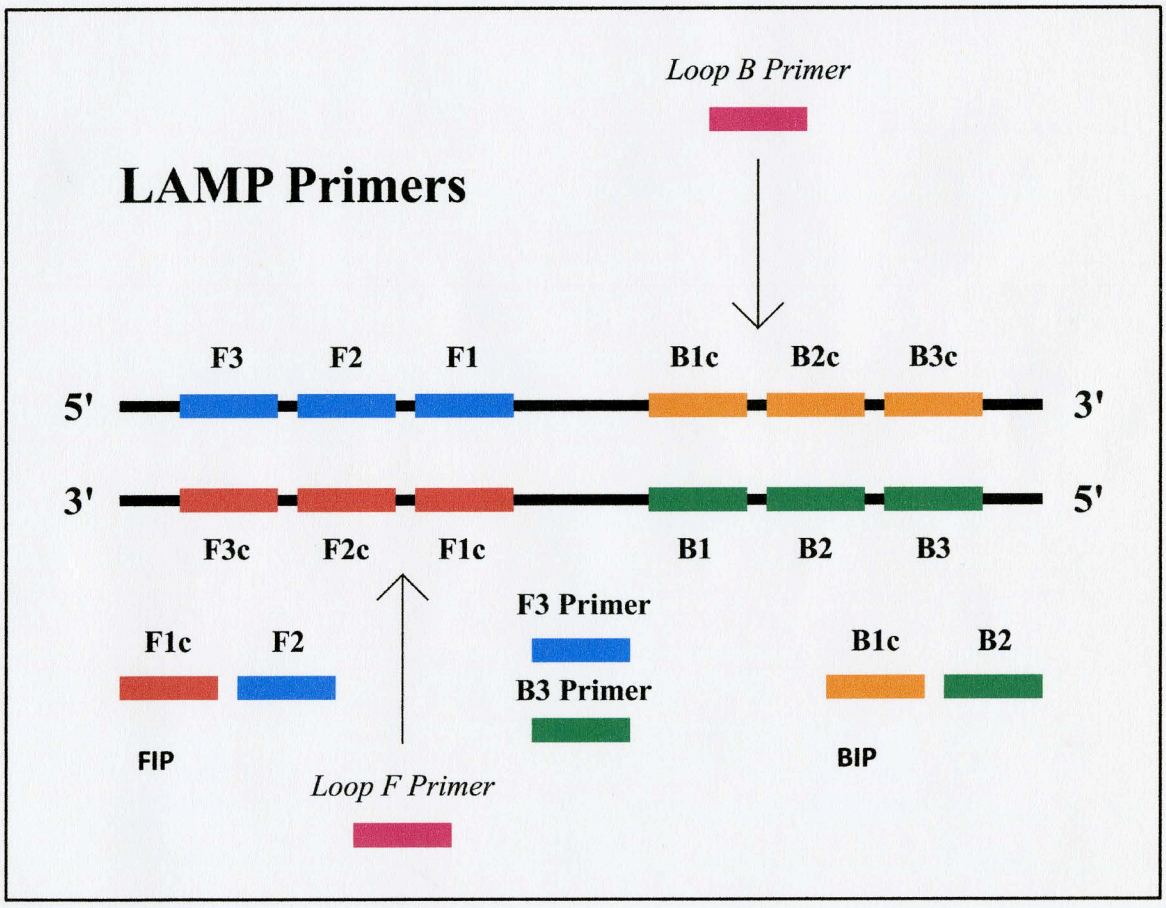

Figure 1.3 – Description of primers involved in LAMP. Four primers detect 6 specific regions. Loop primers increase amplification efficacy. These primers anneal to single stranded loop regions that complementary to the B1 and B2 regions F1 and F2 regions.

Design parameters for LAMP primers

There are four main factors considered when designing LAMP primers. These factors include melting temperature or Tm of the primers, stability at the end of the primers, GC content, and secondary structure (Notomi et al. 2000) .

Primer Tm is subjected to the primer's GC or AT densities in its sequence. GC rich regions should be around 60–65◦C. AT rich regions should be around 55–60◦C.

The ends of the primers must have a certain degree of stability because DNA synthesis begins at endpoints of the primers. This should occur such that the exchange of free energy is exergonic. An exergonic reaction is a chemical reaction where the change in the Gibbs free energy (ΔG) is negative. The more negative the ΔG, the more often the primer will anneal to the template.

A primers GC content should be about 40% to 65%. Specifically, primers with a GC content ranging between 50% and 60% should be used.

To prevent primer-dimer product formations, all primers should not form secondary structures.

Target regions can be found from online data bases such as GenBank (www.ncbi.nlm.nih.gov/genbank/) or Ensembl (www.ensembl.org) where genomic regions are readily available for a variety of organisms, including humans (Benson et al. 1996), (Birney 2003) . The software known as Primer Explorer ver. 4 (http://primerexplorer.jp/elamp4.0.0/index.html), is a LAMP primer designing software

used to sequence all primers complementary to the target-regions. The software also assists in optimization of the primers by using a combination of design parameters. Such software is used to virtually create and evaluate LAMP primers, and is available free.

Lab-on-chip methodology

Introduction

There is a great interest in the use of lab-on-chip (LOC) technology as a diagnostic tool in the health-care sector (Yager et al. 2006) . The lab-on-chip allows rapid, automated integration of many laboratory processes onto a single platform. Such devices can decrease the turnaround time of analysis to a few minutes. The chips range in sizes from a few square millimeters to centimeters, and can accomplish a variety of on-board functions from sample preparation to detection. Other lab-on-chip applications include forensics (Liu et al. 2011) , flow-cytometry (Cheung et al. 2005) , and electrophoresis analyses (Lagally et al. 2001). Small sample and reagent volumes are normally used on these platforms which reduce cost and increase the response to analysis time since fluids are diffusing over very short distances. The chips are often manufactured from a variety of materials including polymers, silicon, glass, or a combination of these. Lab-on-chip devices use small channels to control fluid flow. They may also include controlled micro pumps (e.g. dose-controlling) or valves (e.g. unidirectional flow and/or isolation). Sensing capacity is often provided from electrochemical or optical sensors external or integrated into the chip (Kirby 2010).

Nucleic acid amplification on lab-on-chip

The advent of LOC technology has allowed integration of nucleic acid amplification. These chips often perform a majority of necessary steps for "sample-to answer" detection, such as nucleic acid isolation, amplification and transport all at the level of the chip.

Berkel et al. developed a novel microfluidic platform that combined sample preparation with impedance cytometry in a single chip for POC blood cell analysis (Han et al. 2012). The platform used a small amount to blood, incorporating lysis and quenching of the blood-sample, to provide full sample preparation. Dedicated channel networks were constructed each specific for the component of blood to be analyzed. The chip provided a 3-part differential, platelet/erythrocyte counts as well as haemoglobin concentration measurements using this methodology.

Easley et al. described the design of an integrated glass microfluidic analysis system for detection of *Bacillus anthracis* (anthrax) from whole-blood samples (Easley et al. 2006). The system conducted DNA extraction/purification, PCR amplification and electrophoretic separation for detection. An elastomeric membrane, together with directed flow, focused and purified the DNA.

Perhaps one of the major drawbacks to all the previously mentioned systems is a dependence on separate interconnected chambers/modules to accomplish on-chip nucleic acid purification/isolation, amplification, and detection. Furthermore, such platforms may require complex microvalves and/or pumping methods to manipulate fluid flow from one

chamber to another increasing chip complexity and cost. Several researchers have eliminated this obstacle by using a single chamber.

Lee et al demonstrated DNA extraction and amplification in a single chamber with detection provided by a hand-held, portable laser diode and irradiated magnetic beads. This is collectively known as laser-irradiated magnetic bead system (LIMBS), (Lee et al. 2006). The presence of E. coli, gram-positive bacterial cells, and hepatitis B virus mixed with human serum was detected with the platform.

Kim et al described an integrated, polycarbonate microfluidic chip that utilized a single PCR chamber coupled with a nanoporous aluminum oxide membrane (AOM) to isolate, amplify, and detect both DNA and RNA target-sequences at the level of the chip (Kim et al. 2010). Target-samples were mixed with a lysing agent before introduction, and nucleic acids where then bound to the membrane. The AOM was highly resistant to liquid flow and required a specialized flow control algorithm. The membrane was delicate and required special handing.

Isothermal nucleic acid amplification eliminates the requirement for a thermocycler. This can be advantages for POC applications because there is less need for complex thermal control.

For example, Tourlousse et al recently developed a LAMP-based disposable polycarbonate microfluidic chip for quantitative detection of various pathogens, by using an array of interconnected reaction wells (Tourlousse et al. 2012). LAMP primers were dehydrated and pre-stored inside the chip, with a single pipetting step to dispense the

sample.

Lutz et al. demonstrated, for the first time, a lab-on-a-foil system that offered automated analysis of nucleic acids that was based on isothermal recombinase polymerase amplification or RPA for detection of *staphylococcus aureus* strains

(Lutz, Weber et al. 2010). The system consists of a foil-based centrifugal microfluidic cartridge. The cartridge integrated pre-stored reagents and a commercial centrifugal analyzer. The centrifugal analyzer allowed incubation and real-time fluorescence from tagged products.

Fang et al. described a LAMP portable integrated microchip for detection of various bacteria strains (Fang et al. 2012). The chip performed DNA amplification using a single chamber with results visualized by the naked-eye in a single or multiplex assay format.

Liu et al. developed a polycarbonate single-chamber LAMP-based microfluidic chip that enabled nucleic acid purification (Liu et al. 2011). The chip integrated an inexpensive, easy to implement, Flinders Technology Associates isolation membrane (Whatman, FTA) for HIV detection from oral fluids. It could also be used for DNA detection from E. coli and was effective at isolating the nucleic acids from samples. The membrane contains chemicals, attached to a fiber matrix, that lyse cells, denature proteins and protect nucleic acids from nucleases, oxidative, and UV damage (Whatman, FTA).The membrane also aided in removal of air bubbles prior to amplification.

Thermal-responsive valves

A majority of the previous devices mentioned do not use valve technology. Often, groups will use a temporary solution (Kim et al. 2010), (Liu et al. 2011), e.g. transparent tape placed at the inlet and outlet ports to seal off the chip. Several valveless mechanisms have been developed to minimize evaporation (Zhang et al. 2010) , however, sealing the reaction chamber is a robust way of reducing both contamination and evaporation.

Current valve systems incorporate a variety of innovative mechanisms such as magnetic (Pamme 2005), piezoelectric (Shao et al. 2004), thermal (Wang et al. 2005), (Chen et al. 2008), mechanical (Galambos et al. 2011), hydrolytic, and pneumatic systems (Thuillier et al. 2005). In general, these valves are often smaller versions of their conventional macro-valve counterparts, having the same complex moving mechanical and/or electrical actuating components (Oh et al. 2006) . The micro-valve version, therefore, usually requires complex three-dimensional structures and assembly. The valves may also require an off-chip power-supply and/or computer.

Recently, the use of thermal-responsive valves has offered inexpensive and simpler alternative to other valve technologies. Thermal valves generate motion by thermal expansion and do not require moving parts. A combination of inexpensive materials are used to accomplish thermal amplification such as PDMS, and have been incorporated into microchips because of their low-cost, reproducibility and simple designs.

For example, Wang et al demonstrated the use of thermally sensitive, PDMS, hydrogel valves that were integrated onto a polycarbonate microfluidic chip (Wang et al. 2005) .

To test the utility of the valves, a PCR chamber, embedded on the chip, was sealed under a pressure of 200 kPA. The hydrogel-valves did not require any moving parts and were biocompatible.

In RT-LAMP, because amplification products are generally not handled after the reaction, single-use valves may be desirable because there is no requirement for continued actuation.

Carlen et al.'s group developed a micro-valve from paraffin wax films for microfluidic applications. The paraffin wax substrates melted on heating and sealed a PCR reaction chamber (Carlen et al. 2004). However, fabrication of the valves required surface micromachining to place the valves onto the chip.

Notably, Samel et al. reported on the use of easy to fabricate, thermal valves for microfluidic applications (Samel et al. 2007). The valve consisted of a composite of PDMS mixed with Expancel microspheres. Upon heating to 80 degrees Celsius, the microspheres underwent irreversible expansion with a volume increase of 270% of its original volume.

Expancel® microspheres are small spherical polymer shells with diameters of around 10 μm. The shell is comprised of a copolymer of monomers such as vinylidene chloride, acrylonitrile, or methyl methacrylate. The spheres are filled with a small amount of pressurized liquid hydrocarbon (e.g isobutane or isopentane). During heating, the outer shell undergoes plastic deformation while the internal liquid hydrocarbon phase changes into a gas. A dramatic volume increase is observed, where the sphere diameter increases

up to four times its size with a volume increase of a factor of six. The process is irreversible since the thermoplastic shells harden upon cooling. The microspheres have been shown to have no negative effects after expansion when used with a variety chemicals and solvents (Griss et al. 2002).

Samel et al demonstrated the valves on a lab-on-chip polymer-based platform that used a novel DNA sequencing technology called Pyrosequencing (Ronaghi et al. 2001) combined with real-time detection. Nanoliter fluidic handling of fluid was accomplished in which the expansion of the valves provided directional flow, displacing the liquid through a microchannel into a reaction chamber (Roxhed et al. 2006) .

Later, Liu et al used the PDMS valves on a polycarbonate-based microfluidic chip (Liu et al. 2011), where the thermal-responsive valves effectively sealed a LAMP reaction chamber. However, there was no on-chip nucleic acid purification/isolation, limiting its usage in the laboratory setting.

Several design factors are important in producing PDMS-expandable valves that will form a hermetic seal. Notably, the concentration of microspheres added to PMDS, determines the level of compliance of the thermoplastic valves, and hence ability to form an airtight seal (Samel et al. 2007). As such, an increase in the concentration of microspheres will increase the expandability of the composite valves. If too many microspheres are added, then the relative viscosity of the solution increases and the valve loses the ability to form a seal. Additionally, the volumetric ratio of the PDMS base to the curing agent significantly alters the expandability of the valves. In such a case, a decrease

in the concentration of PDMS curing agent results in an increase of the elasticity and the expandability of the composite. For example, an expansion of nearly 550% upon heating results from a mixture of 25% microspheres by volume with a 20:1 ratio of PDMS to base/curing agent (Liu et al. 2011). Therefore, a trade off of compliance to expandability is observed. A mixture of 5% microspheres (by volume) to a ratio of 20:1 PDMS base to curing agent resulted in a matrix that expands 150% +/- 5% upon heating. This was found to form an adequate seal.

Another important characteristic of the thermal-responsive valve is that they do not heat uniformly because of the low thermal conductivity of PDMS (0.15 W/m-K), (Mark 1999). As a result, microspheres that are adjacent to heat will undergo expansion first. Microspheres that are further away from a heat source will expand later (Figure 5.1 A, B). Integrating thermal conductive materials (e.g. silver powder) materials into the PDMS composite is one way of increasing the thermal uniformity of the valves.

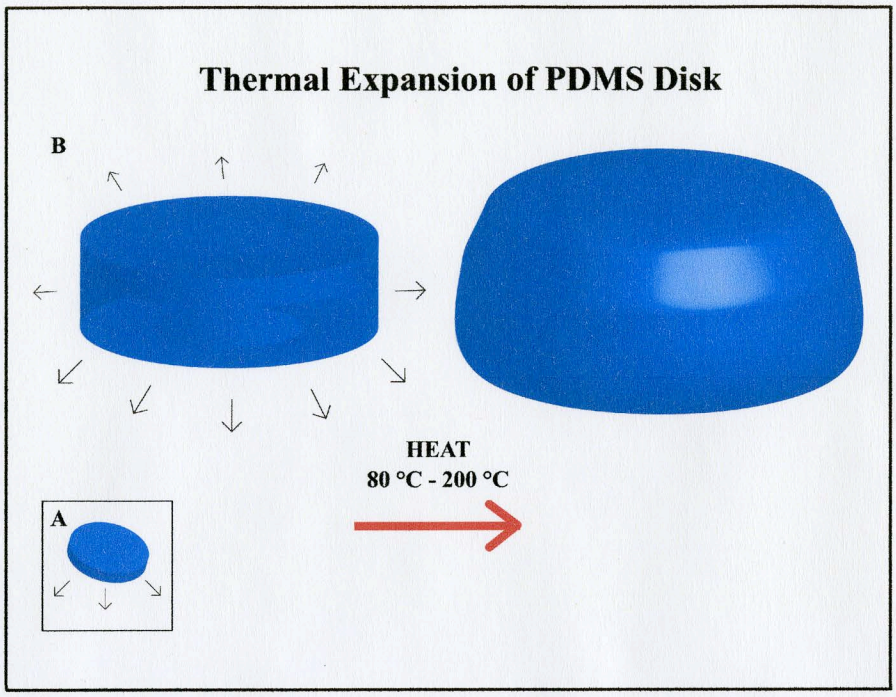

Figure 1.4 – A: Greater expansion is observed when adjacent to a heating element. This is because surface microspheres expand first compared to microspheres that are further away. B: Three-dimensional render of the valve before and after expansion

DESIGN AND METHODS

LAMP cassette design

The LAMP cassette was constructed from a 6.00 mm polycarbonate sheet, and two 250μm polycarbonate films (McMaster Carr,GA, USA). The completed cassette is 35 mm in length, 35 mm in width, and 6.5 mm in depth (Figure 2.1).

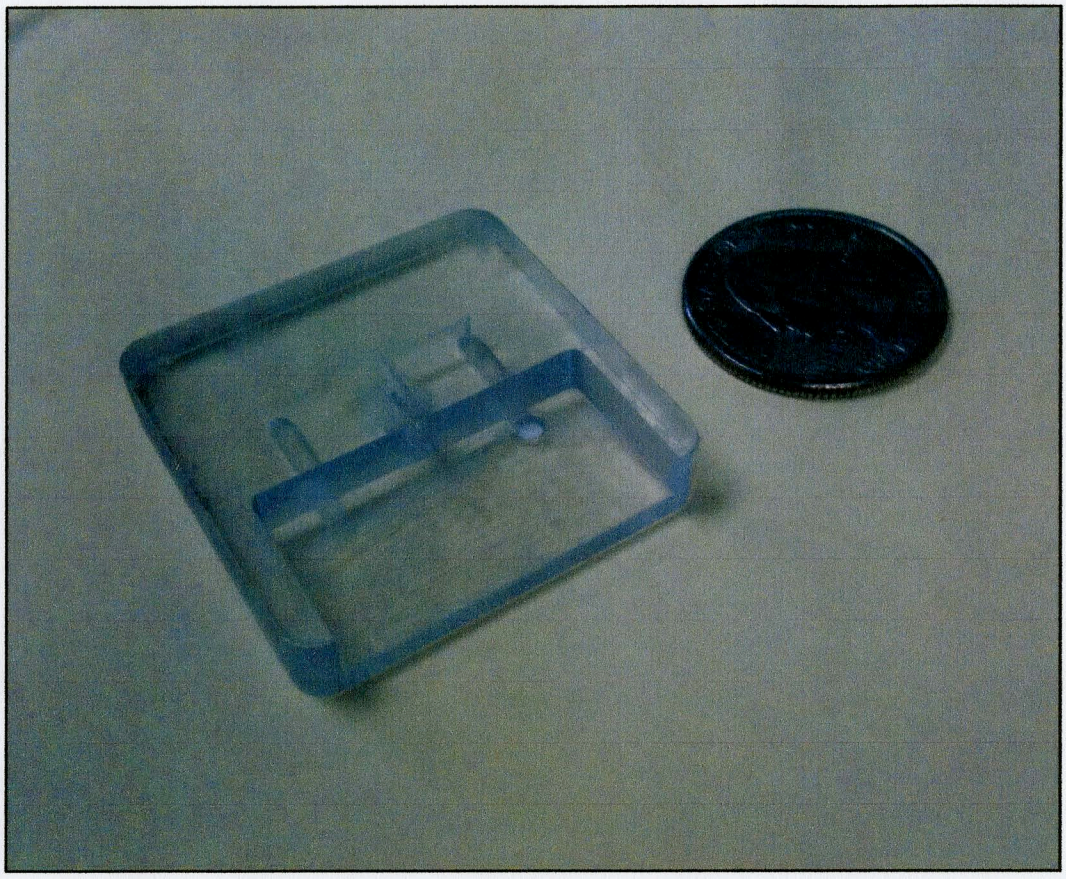

Figure 2.1 – Image of completed cassette. The cassette is comprised of three pieces of polycarbonate: two films and one center-sheet. The chip's features are machined on the center-sheet. The films are solvent bonded to the center-sheet using pure acetonitrile.

The 250μm think polycarbonate films are located on the top and bottom of the main-body, polycarbonate sheet. Figure 2.2 gives an exploded view of the cassette.

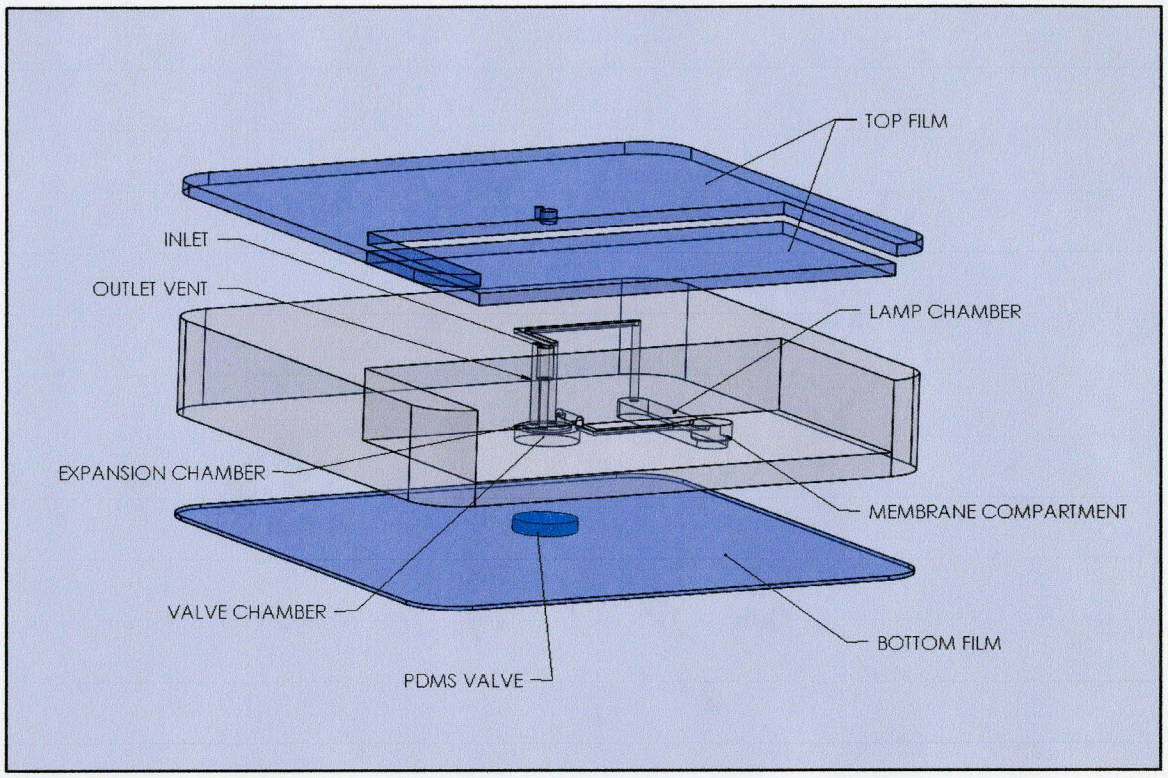

Figure 2.2 – Exploded view of the cassette.

The films were cut to size using a CO_2 laser guided machine. Both films were solvent bonded to the main-body using pure acetonitrile at room temperature. Residue was removed by heating the cassette to 50 degrees Celsius for 12 hours.

The cassette is comprised of 4 functional domains: the reaction (incubation) chamber, valve chamber, inlet and outlet channels, and membrane compartment (Figure 2.3). The cassette body was milled using a precision, computer controlled (CNC) milling machine. The reaction chamber is 10 mm in length, 2.0 mm in width, 1.0 mm in depth, and has a 20 μL in volume.

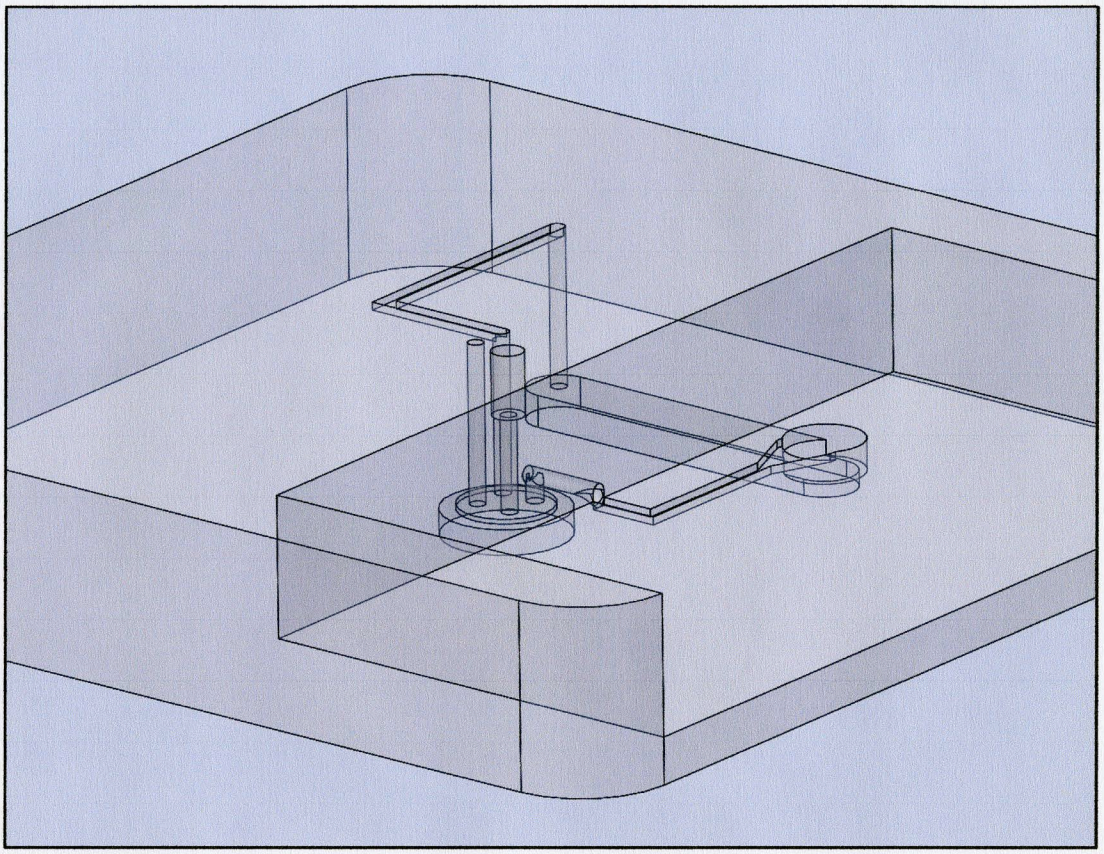

Figure 2.3 - The cassette encompasses a multi-depth channel network to allow flow of sample into LAMP chamber. The FTA membrane provides unidirectional flow of fluid.

The reaction chamber was machined on the bottom of the main-body. To form the inlet and outlet channels, 0.05 mm wide by 0.25 mm deep, rectangular cross-sections were machined on the top of the main-body. The inlet-port travels through the depth of the main-body to connect to the membrane chamber. To gain access to the inlet port, a 0.8 mm diameter hole was drilled with a table top milling machine through the top of the main-body and film. Similarly, to gain access to the outlet vent, a 0.5 mm diameter hole

was drilled through the top film.

To form the FTA membrane compartment, a 2.5 mm diameter hole, 1 mm deep, was machined from the top of the cassette. A 2 mm diameter hole was then drilled from the bottom through the LAMP chamber, concentric with the top hole. Figure 2.4 shows a view of the membrane compartment. The thickness of the FTA membrane is 500 um. The membrane is placed into the 1 mm deep compartment and supported by an edge created by the difference of diameters of the top and bottom hole cuts.

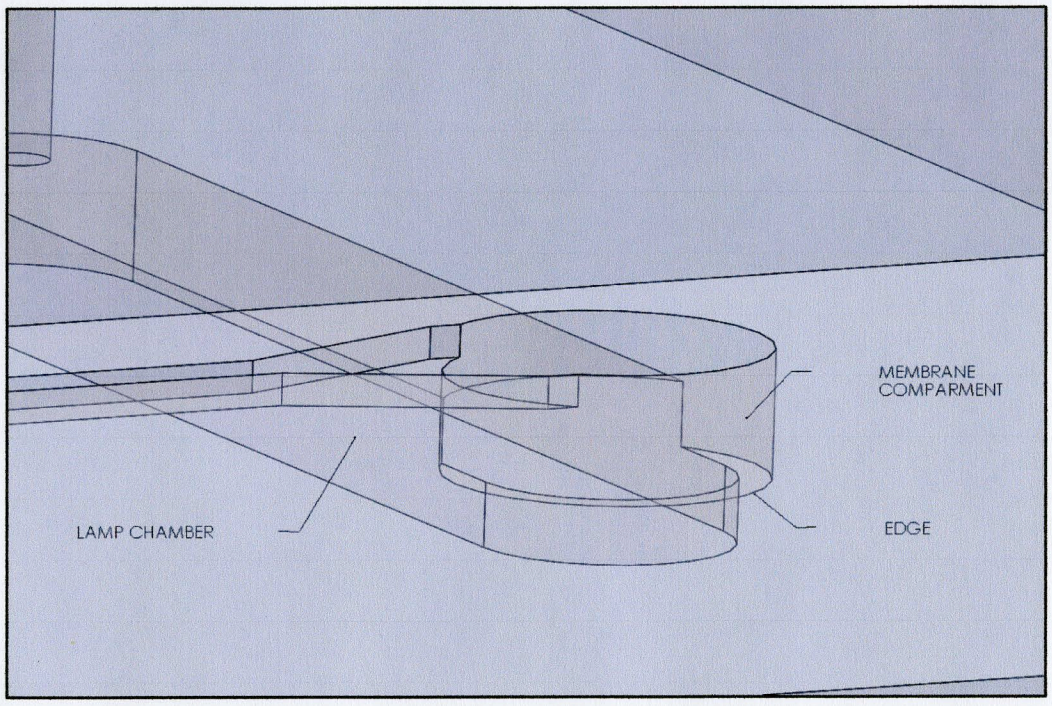

Figure 2.4 – The FTA membrane compartment is supported by an edge machined onto the center-sheet. FTA membrane is placed in the flow path of the sample.

The valve chamber was machined on the bottom of the cassette. Figure 2.5 gives a cross section view of the valve chamber.

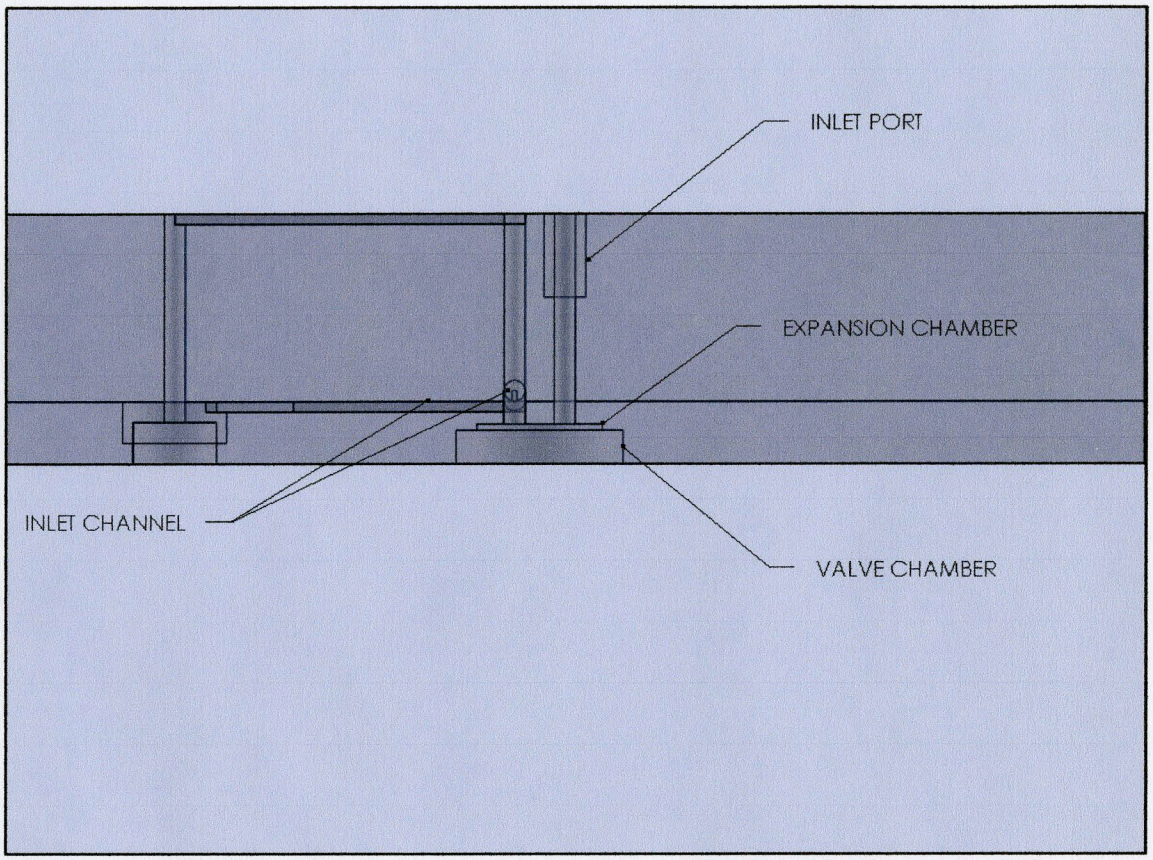

Figure 2.5 – Cross-sectional view of valve chamber. The PDMS valve expands into the expansion chamber sealing the reaction chamber and all the channels.

The valve chamber consisted of a 4 mm diameter, .08 mm deep well to hold the thermoplastic valve. A 3 mm diameter, 0.35 mm deep expansion compartment was machined directly above the valve compartment to allow fluid transport when the valve is in its open state.

When the valve is not expanded, fluid is able to flow across the expansion compartment to the reaction chamber. During heating, the thermoplastic valve expands and seals the conduits.

Thermal-responsive valve design

Thermal-responsive valves were formed from a mixture of PDMS (Sylgard 184 Silicone Elastomer Kit, Dow Corning) and dry unexpanded microspheres (Expancel 031 DU 40, Duluth, GA). The temperature range over which the valve undergoes expansions is 80 to 120 degrees Celsius. Each valve is 4 mm in diameter and 0.8 mm thick. The mixture was made with 5% microspheres (by volume) using a ratio of 20:1 PDMS base-to-curing agent. A polycarbonate mold consisting of 4 mm diameter, 0.8 mm deep wells was machined (Figure 2.6), in which the polymer suspension was dispensed into valve molds. A 5 mm think flat polycarbonate slab was placed over the PDMS-expandable microsphere suspension. Figure 2.8 shows the valve installed in the chip.

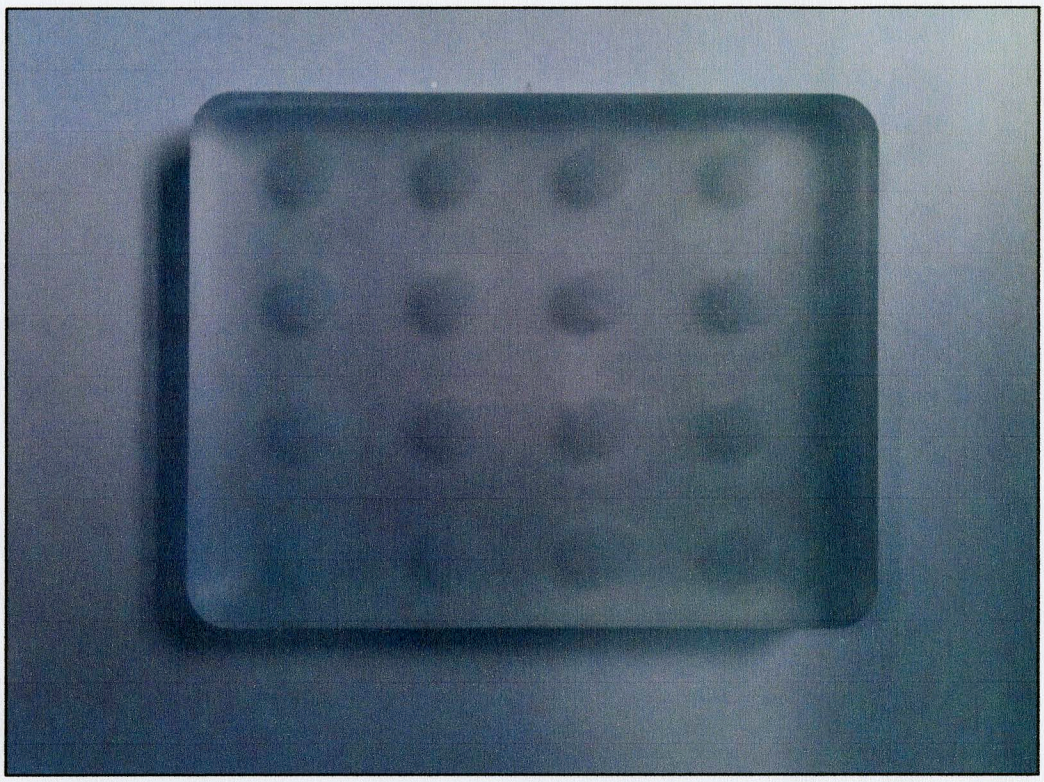

Figure 2.6 – Polycarbonate mold used to form PMDS-valves. The composite is dispensed into the mold and covered with a top plate.

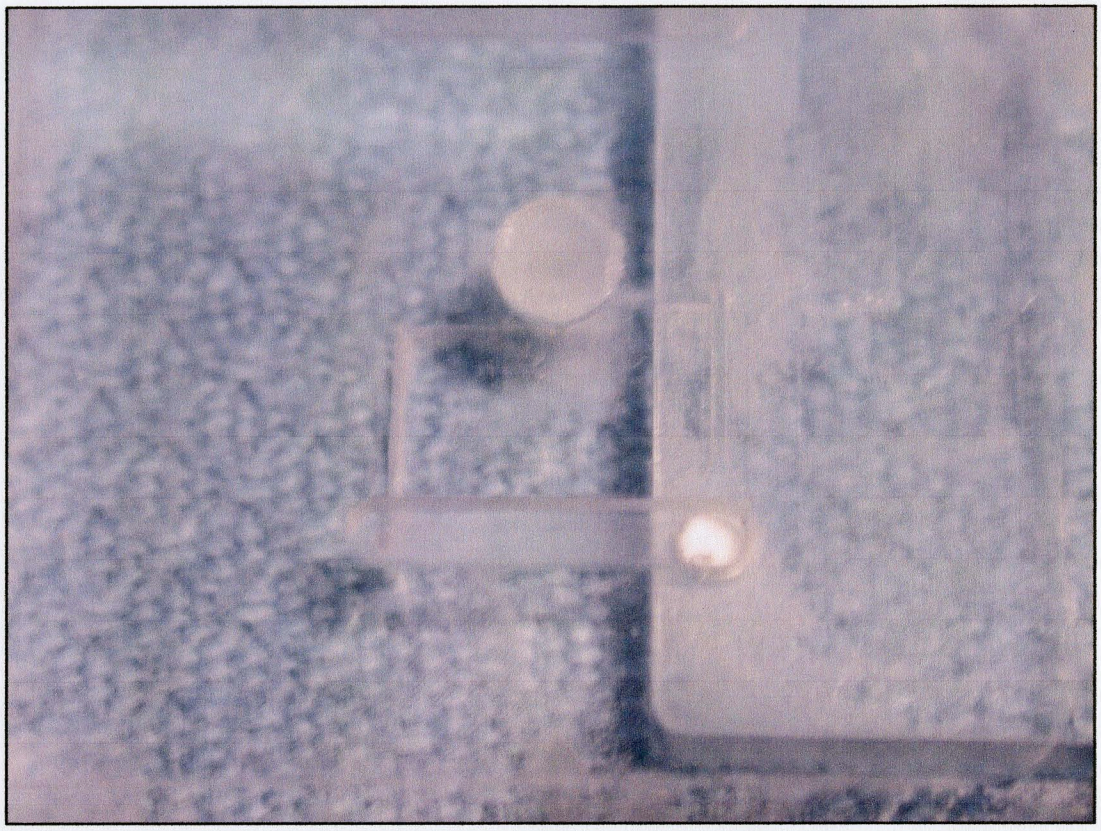

Figure 2.7 – Bottom view of the chip with PDMS valve installed. When heated the valve expands and seals the reaction chamber.

Operation of the cassette

A sample is mixed with a lysing agent and introduced into the inlet-port with a syringe. The lysed sample filters through the FTA membrane. The membrane intercepts nucleic acids before they reach the reaction chamber. Nucleic acids are eluted from the membrane by washing twice with a wash buffer. At this point, an inhibitor removal buffer is introduced into the chip to remove any amplification inhibitors present in the sample. The cassette is then air-dried for 30 seconds. Once wetted, the membrane expands, and provides unidirectional flow of fluid. This prevents any fluid from entering

into the inlet-channel. Waste is discarded through the outlet.

A LAMP master mix and SYTO® Green 9 dye is then syringed into the chip. The LAMP master mix contains LAMP primers and necessary reagents. Once this occurs, the thermal-responsive valve is heated to 80 degrees Celsius.

Thermal control is provided by a battery-powered polyimide-based thin-film heater located directly beneath the valve compartment on the outside the bottom film. Subsequently, the thermoplastic disk undergoes expansion and seals the chamber and channel networks. Following closure of the valve, the LAMP chamber will heat to 65 degrees Celsius for 1 hour using the same heater. Real-time LAMP detection will be carried out using a potable optical detection system such as the ESE optical detection system (Fluo Sens SD 523, Qiagen Lake, Germany). The device provides fluorescent excitation and detection using a 470 nm LED as an excitation light source and Si-photodiode for fluorescent detection.

EXPERIMENTAL

Research design and rationale

The valve was tested to seal the LAMP chamber. The objective of the valve is to reduce contamination and evaporation as a consequence of exposure to the environment. There is a risk of contamination due to high concentration of amplified products. Evaporation occurs because of the elevated temperatures used to heat the LAMP reaction. The valve reduces these effects by isolating the LAMP reagents and sample in the reaction (incubation) chamber in a "single-shot" closure by thermal expansion. The process is irreversible. A temperature of 80 degree Celsius is required for expansion of the valve. During LAMP, the reaction normally proceeds under isolated (i.e sealed) conditions the way it would in a reaction vial. Under these conditions, isolated PCR reactions cause an average pressure of 200 kPa (Wang et al. 2005). In order to effectively seal the chamber, the single thermal-responsive valve must be able to withstand pressures up to 200 kPa without any leakage. A failure pressure is reached when the valve can no longer withstand the pressure and leakage occurs, or the valve is displaced from its original position.

Methods

Valves were fabricated using a composite of PDMS and expandable microspheres. A ratio of 20:1 PDMS base-to-curing agent was mixed with 5%, by volume, of 031 DU expandable microspheres. The composite was degassed in a vacuum to remove any air bubbles that may have formed. The composite was pipetted into the valve mold. A 5 mm slab was carefully placed over the valve-mold. Surface tension pulled the polycarbonate slab into contact with the mixture without forming any air bubbles at the interface. The pre-polymer composite was left overnight and then heated to 60 degrees Celsius for 4 hours. This temperature was below the phase transition temperature of the microspheres in order to cure the PDMS disks. Once cured, the valves were removed using a scalpel.

To test the response time of the PDMS disk, ten disks were individually placed on a 250 μm polycarbonate film, and heated to 85 °C using a heating block. The valves were measured before and after expansion using a caliper. Lateral and vertical measurements were collected.

A single valve was installed into the valve compartment. Thermal control was provided by a thin-film polyimide heater that was battery-powered. The thin-film heater required a voltage of 28 Volts (V) and 5 Watts (W) of power. The heater had a resistance of 157 Ohms (Ω).

The current in amperes (A) was calculated using Ohm's law:

$$I = \frac{V}{R};\ \frac{28\,V}{157\,\Omega} = .178 \cong .18\,A.$$

Three 9V batteries were used to power the heater. This found was by dividing the operating voltage by 9 V, 28V / 9V = 3.11.

Each battery had a voltage of roughly 9.15 V. The total voltage supplied to the heater was 27.45 V. This was sufficient to power the heater. A single pole-single throw (SPST) toggle switch was used for on and off control. The experimental set-up is shown is Figure 3.1.

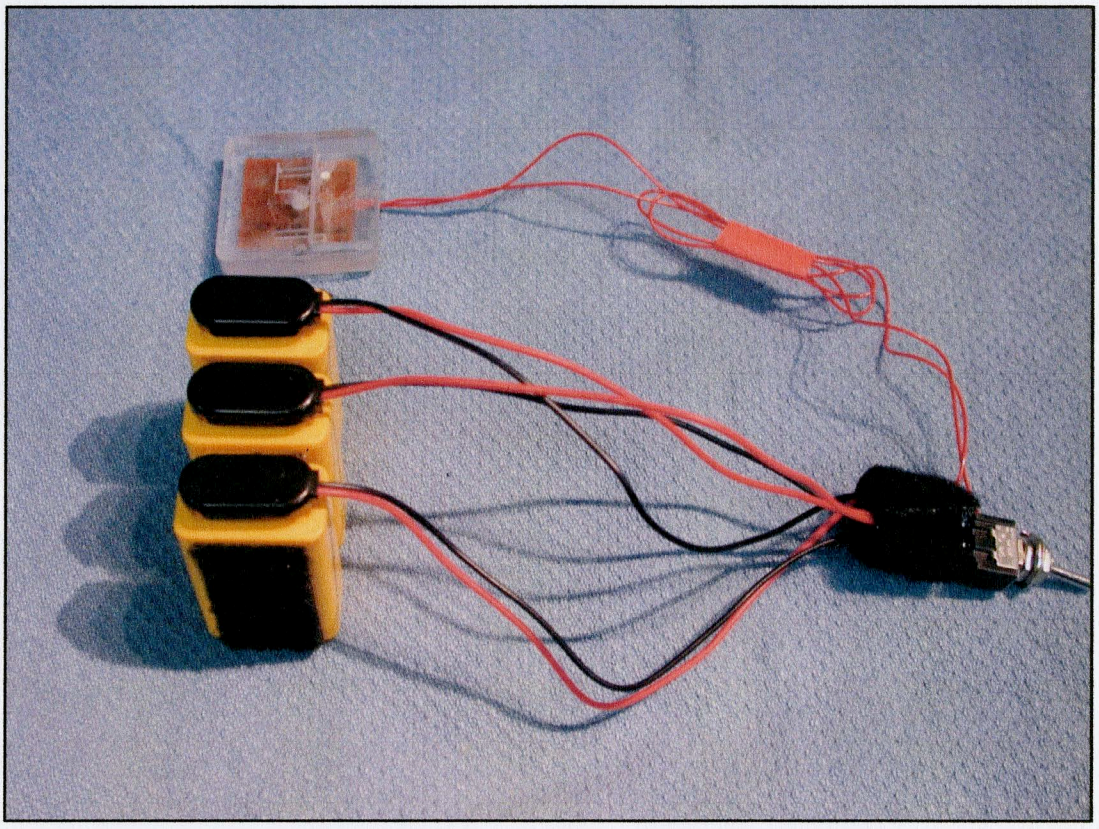

Figure 3.1 – A battery powered thin-film heater is placed underneath the cassette. Three 9V batteries were used to power the heater.

Temperature was controlled in open loop mode and was monitored with a thermocouple placed directly against the portion of the heating element of the thin-film heater. The thermocouple reading was monitored on the LED display of a DMM using the volt meter setting.

To test the holding pressure of the valve, pressure was measured using a 50 µL air-tight syringe inserted into the cassette at the interface between the inlet channel and inlet-port (Figure 3.2 A, B, and C). The syringe needle punctured through the top film.

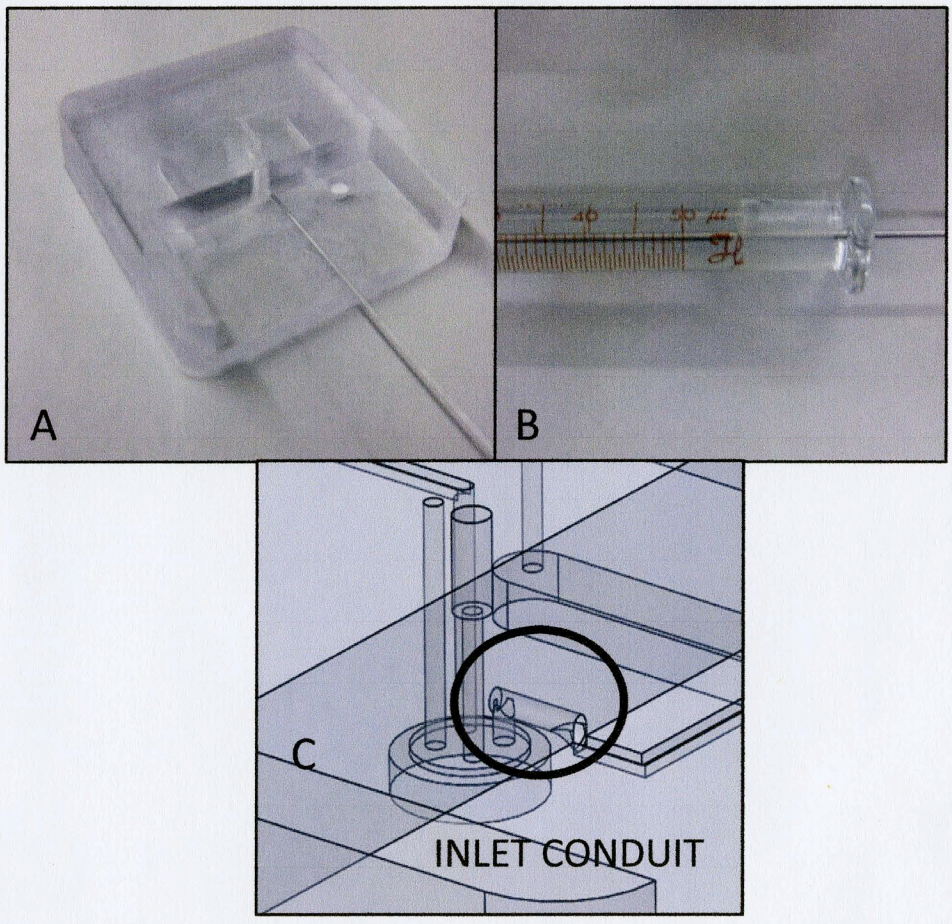

Figure 3.3 – A: Image of cassette with inserted syringe. B: A 50 µL air-tight syringe was used. Each 1 µL interval was equal to 1 mm. C: Close-up of inlet-conduit.

The ideal-gas law was used to estimate pressure. The compressed volume upstream of the valve was found by subtracting the volume of air introduced by the syringe from the volume of air in the inlet-conduit. The displacement volume of the syringe was measured with an electronic caliper. Each 1 µl interval on the syringe was equal to 1 mm.

Below is the calculation to estimate pressure:

$$P_1 \times V_1 = P_2 \times V_2$$

V_1 = Volume of inlet conduit.

$$V_1 \approx 8.6 \times 10^{-4}\, ml$$

$V_{syringe}$ = Volume displacement of the syringe.

$V_2 = V_1 - V_{syringe}$ = Volume of compressed air upstream of the valve.

$$P_1 = 1\ atm$$

$$P_2 = \frac{P_1 \times V_1}{V_1 - V_{syringe}} \times \frac{101.3\ Kpa}{1\ atm}$$

The cassette was monitored for signs of leakage and/or displacement of the PDMS disk from its original position.

RESULTS

A disposable, portable polycarbonate cassette was designed and fabricated. The cassette allows loop mediated isothermal amplification inside of a single reaction chamber. The reaction chamber is sealed using a self-actuating, thermally-responsive valve. A FTA membrane was installed with the expectation of providing isolation of nucleic acids from a sample.

Figure 4.1 A-D shows vertical and lateral measurements of a PDMS disk before and after expansion.

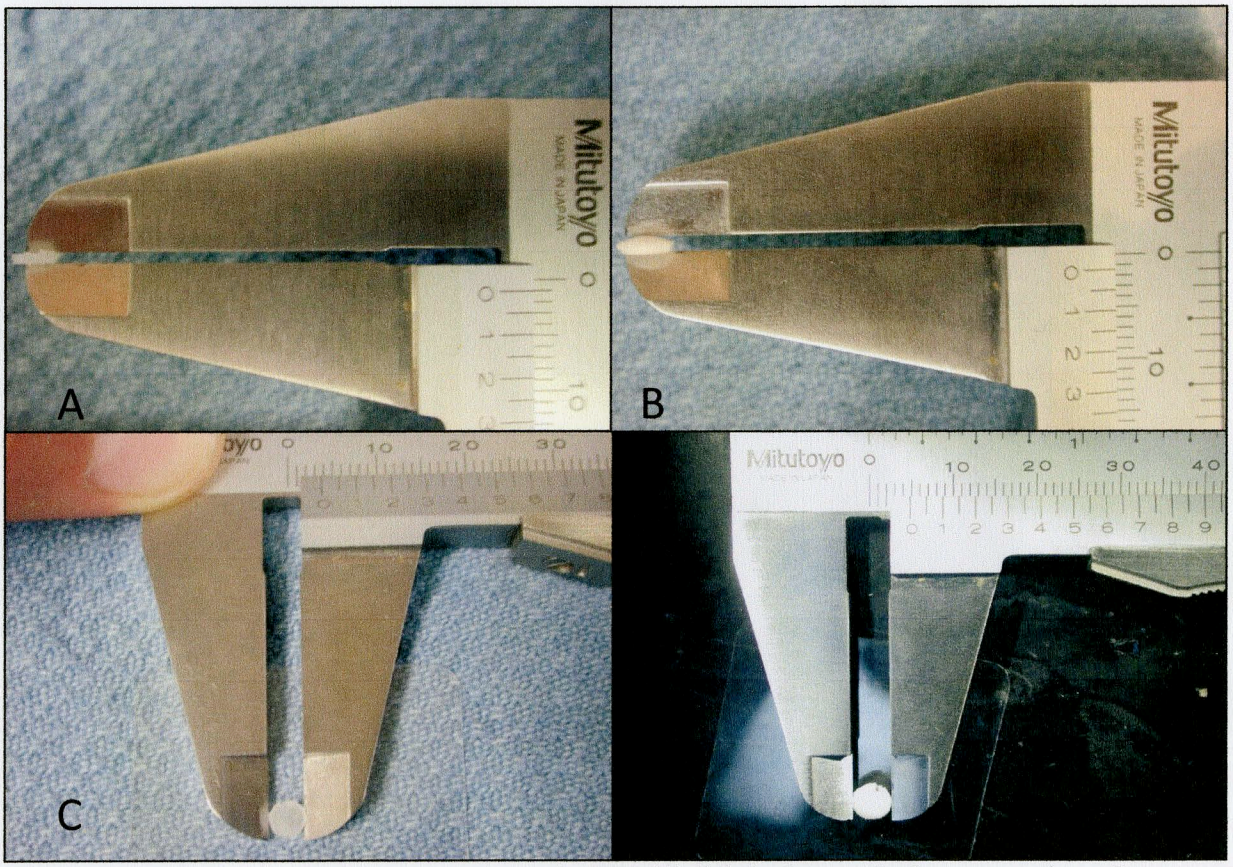

Figure 4.1 – A-B: Vertical measurement of a PDMS disk before and after expansion. C-D: Lateral measurement before and after expansion.

Table 4.1 is a summary of all measurements collected.

Diameter Before (mm)	**Diameter After (mm)**	**Percent Increase (%)**	**Thickness Before (mm)**	**Thickness After (mm)**	**Percent Increase (%)**
3.9	4.9	**25.6**	0.9	1.5	**66.7**
3.8	4.7	**23.7**	.08	1.3	**62.5**
4.0	4.9	**22.5**	.07	1.3	**57.0**
3.9	5.0	**28.0**	.08	1.6	**62.5**
3.7	4.7	**27.0**	1.0	1.7	**60.0**
3.9	4.9	**25.6**	.08	1.5	**63.0**
4.1	5.0	**22.0**	0.9	1.5	**66.7**
3.8	4.7	**23.7**	1.0	1.6	**60**
4.0	4.9	**22.5**	1.1	1.7	**63.6**
4.2	5.1	**21.4**	1.2	1.8	**66.7**

Table 4.1 – Summary of measurements collected from ten PDMS valves.

The average disk diameter before expansion was 3.93 +/- 0.14 mm. After expansion, the average disk diameter was 4.9 +/- 0.14 mm. Disk diameter increased 24.2 +/- 2.17 percent.

The average disk thickness before expansion was 0.92 +/- 0.15 mm. After expansion, average disk thickness was 1.5 +/- 0.27 mm. The thickness of the disk's increased 62.8 +/- 3.05 percent.

The average time for expansion of the ten PDMS disks was 8.6 +/- 0.92 seconds.

The valve inside the cassette underwent expansion when heated to over 85 °C. Figure 4.2 shows the expanded valve in the cassette.

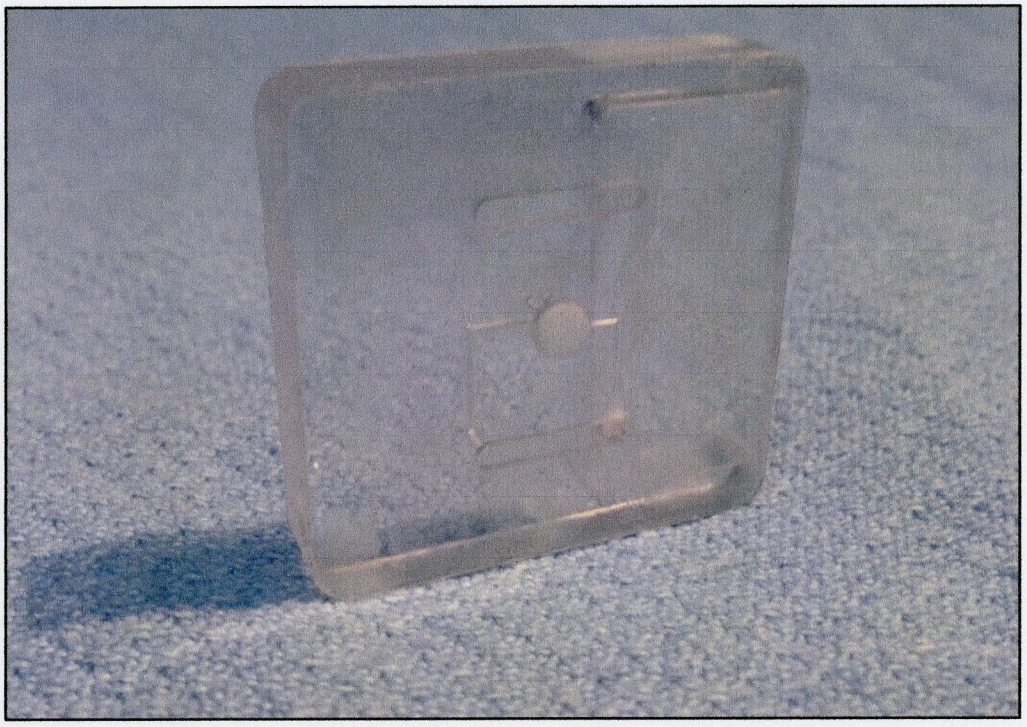

Figure 4.2 – Image of expanded PDMS disk within the cassette.

Figure 4.3 - The experimental set-up used to measure the volume displacement from the air-tight syringe.

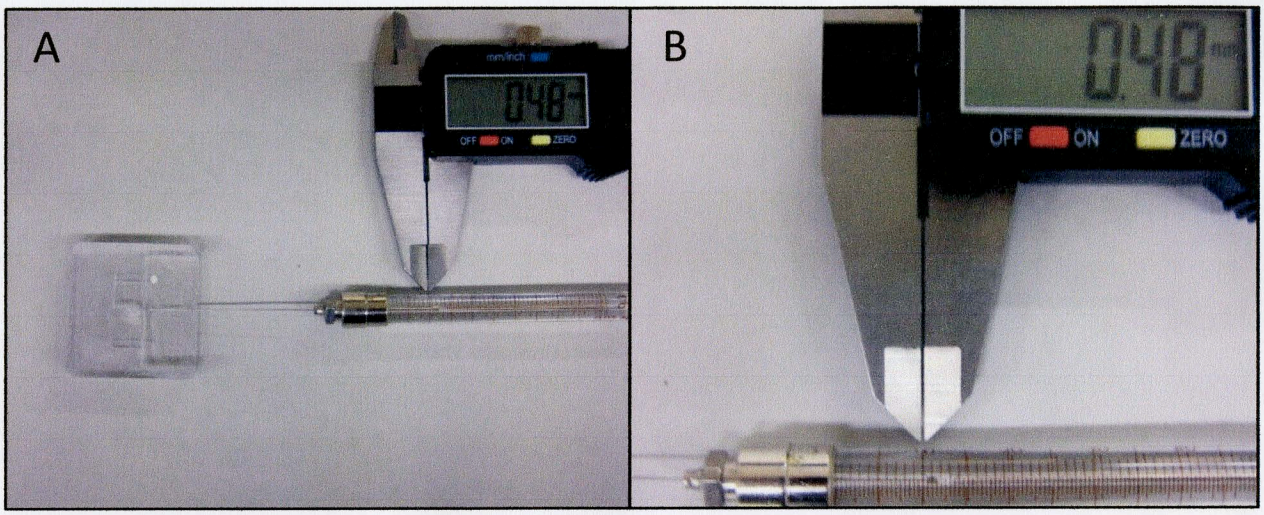

Figure 4.3A-B: The displacement of the syringe was taken with an electronic caliber.

The estimated pressure applied to the valve was, $P_2 \cong 233 +/- 5.0\ Kpa$.

Five measurements were collected for $V_{syringe}$: 0.48, 0.49, 0.48, 0.50 and 0.48 mm.

An example calculation using one of the values for $V_{syringe}$ is shown below:

$$P_2 = \frac{1\ atm\ \times 8.6\ \times 10^{-4}\ ml}{8.6\ \times 10^{-4}\ ml - \mathbf{0.48\ \times 10^{-3}\ ml}} \times \frac{101.3\ Kpa}{1\ atm}$$

The valve inside the cassette withstood a pressure of 233 +/− 5.0 kPa without any leakage.

DISCUSSION

The vertical expansion of the PDMS disk was larger than the distance of the cassette's expansion chamber. The vertical distance of the cassette expansion chamber is 0.35mm. The PMDS disk expanded 0.50 mm vertically. The valve could enclose this area. The PDMS disk withstood a pressure around 233 kPa. This meets the pressure requirement for a PCR reaction enclosed in a reaction chamber of comparable size (Wang et al. 2005). The breakdown pressure of the valve was not determined because the polycarbonate cassette de-bonded when the syringe was compressed further.

The three 9V batteries used to power the thin-film heater can be replaced with a single lithium battery that operates at 28 V. There are also 12 V alkaline batteries available (M121 Alkaline Battery, Duracell, US). These batteries are much smaller than 9V batteries. Using three 12 V batteries, a voltage of 36 V would be supplied to the thin-film heater. A resister would be necessary to limit the voltage to the thin-film heater.

Weak solvent-bonding using acetonitrile resulted in formation of a cloudy appearance on the polycarbonate substrates. An alternative way to bonding the polycarbonate would be to apply the solvent at the edges of the substrate, and to areas around the valve and reaction chamber. Following heating, the transparency of the polycarbonate cassette decreases more. This may be a result of thermal activation of the acetonitrile between the interface of the polycarbonate center-body and films.

A bill of material is provided in Appendix A. The total cost of the complete system is $1056.55. The complete system includes the cassette, all reagents, and optical detector

system.

To reduce costs, an alternative to machining the polycarbonate sheets and films would be to use polymer injection molding. This type of manufacturing process could decrease cost significantly, especially if the cassettes were produced on a large scale. Instead of using polycarbonate, using Acrylic (e.g. PMMA) thermoplastic may be possible since it has a lower T_m (160 °C) compared to T_m of polycarbonate (267 °C), (Olabisi 1997) .

To eliminate the requirement of the optical detector, a smart-phone based fluorescent reader can be developed allowing quantitative or even semi-quantitative readings of the LAMP product. Algorithm application software on the smart-phone could provide acquisition of the data, and display results to the user. The mobile-phone could act as a power source for an external heater.

Several groups have already implemented smart-phone based systems as biosensor devices. For example, Yoon et al reported on a Rayleigh/Mie scattering, optimized lateral flow reader on a cell-phone platform. A semi-quantitative thyroid stimulating hormone (TSH) lateral flow immunochromatographic assay (LFA) was used for detection. The assay was read from the cell-phone to provide quantitative TSH results with higher sensitivity then that of a conventional LFA format.

The cell-phone-based platform utilized the existing flash in the cell phone camera as the light source and an inexpensive optical fiber that directed light through a collimating lens which illuminated the LFA. An image processing algorithm written in MATLAB provided color-discrimination and was converted to application software to be used on

the phone (Yoon et al. 2012).

To house the cassette and detect LAMP product, an external attachment needs to be developed that utilizes components for cell-phone based fluorescence excitation and detection. For instance, excitation can be accomplished by an inexpensive LED blue-laser diode powered by a battery. A single inexpensive lens placed directly in front of the existing camera can provide geometric magnification. Varying the focal length of the lens would result in alterations of the field of view (FOV) and could be used to change magnification. Placing the excitation light-source perpendicular to the fluorescence detection path could allow use of a simple plastic absorption filter to create a sufficient dark-field background, without the need for a thin-film interference filter. Imaging-processing software converted to application software for use on the phone could record the intensity of the fluorescent signal as a function of time. Concurrently, this application software could also control duration of heating. Notably, a potential feature of the application software is to allow the results (i.e. presence of target sequence) to be uploaded to a central-server to track emerging epidemics and/or provide epidemic preparedness at a regional or global scale. The main drawback to such a device is thermal damage to the smart-phone from the elevated temperatures required during LAMP. Such a device should use non-conductive materials that isolate heat produced from the thin-film heater(s).

To reduce power-consumption, the temperature at which the valves undergo expansion could be decreased. Decreasing the temperature to 60-65 °C instead of 80 °C may require

less power (W), and eliminate the need for temperature variability of the thin-film heater. Integrating thermal conductive materials into the PDMS-expandable mixture may increase thermal uniformity. A caveat to this is that the addition of thermally conductive materials may have no effect on performance of the valves since there is no difference in valve response time.

For example, the valves only have to expand a relatively small distance (i.e. from valve to expansion chamber) to form a hermetic seal. Therefore, only a fraction of the microspheres are required to expand in order to close the valve (Liu et al. 2011). Valve response time is determined by the rate at which the surface microspheres expand.

In this report, the gap between the valve chamber and the expansion chamber is relatively small but larger other reported designs that use PDMS valves. Therefore, a small thermal amplification of the PDMS disk is enough to form a good seal, but may be large enough to take into effect the increased thermal uniformity of the microspheres. Further investigation of this design alteration, and its effects on valve thermal uniformity, are needed to see if there is a significant impact on valve performance. This could be done by varying the distance the valves must undergo expansion as a function of the response time using the equal mass fractions of silver-doping material.

Integrating pre-stored LAMP reagents would make the cassette more suited for POC applications. Using the methodology explained earlier used to separate SYBR dyes using wax-crystalline capsules, the LAMP reagents could be encapsulated into wax capsules which melt upon heating the LAMP chamber and then release the reagents in solution

allowing their hydration. Paraffin wax capsules may also be used for this purpose as they have a lower melting temperature requirement (Sharma et al. 2005).

Future experimentation is necessary to provide validation of the device. The cassette would need to be used to detect a target DNA or RNA sequence. Detection of E-coli using the cassette is a potential next step. To confirm amplification occurred, the LAMP products will be removed from the cassette and subject to gel electrophoresis.

ADDITIONAL WORK

LAMP primer design of 5HTR1A gene promoter region

Background

There is pressing evidence showing the role of epigenetic changes in the etiology of psychiatric diseases. Specifically, alterations of the Serotonin receptor, 5-HT, through DNA methylation of critical genes in the 5-HT pathway, is shown to be related to major psychopathologies such as Schizophrenia (SCZ), and Bipolar disorder (BDP) (Lesch 1998). DNA methylation is a key epigenetic mechanism that involves the addition of a methyl group (CH3), typically to CpG dinucleotide islands through Cytosine molecules, without changing the DNA sequence. This type of gene regulation can be inherited through cellular division (Jones 2001). However, addressing the role of DNA methylation in epigenetic processes remains a challenge because the brain is inaccessible (i.e. brains samples not normally used for analysis). Therefore, alternative methods of epigenetic analyses are desirable.

Altered states of the 5-HT gene through DNA methylation can be found in peripheral system, in white-blood cells (De Luca et al. 2009) or even red blood cells (Tsujita et al. 1998), (Kuratomi et al. 2008). This occurs because DNA methylation is cell-type selective. As a result, researchers are able to gain a better understanding of the relationship between expression of the neurotransmitter serotonin and DNA methylation from readily accessible cells. Carrard et al reported on increased DNA methylation status of the serotonin receptor 5HTR1A gene promoter in schizophrenia and bipolar patients

using isolated blood leukocytes (Carrard et al. 2011). Wang et al showed that peripheral SLC6A4 serotonin transporter gene and increased levels DNA methylation at particular CpG sites is found in patients with high childhood aggression. They extracted T cells and monocytes from the patients and used immunomagnetic separation to reduce the complexity of the DNA methylation patterns (Wang et al. 2012). The majority of these studies use PCR for amplification of specific 5-HT genes. The PCR primers are designed using online genetic data banks that provide specific regions of the 5-HT genome. Having POC, semi-quantitative or even quantitative detection of 5-HT epigenetic profile to assess brain-function, development, and pathology, is not yet readily available.

To address the challenge, LAMP primers were designed to detect the 5HTR1A gene promoter region. The serotonin promoter region (5HTR1A) was selected as a candidate, given its critical role in brain development and in pathophysiology of SCZ and BPD. 5HTR1A is widely distributed throughout the brain mostly in the cortico-limbic region (Lesch 1999). This will prove invaluable in providing researchers and physicians with more quantitative evidence for diagnoses and/or epigenetic analyses of central 5-HT function. The LAMP primers are designed to be used with the LAMP cassette previously described to allow the possibility of POC diagnostics.

Lamp primer design

Four LAMP primers were designed using Primer Explorer ver 4 (http://primerexplorer.jp/elamp4.0.0/index.html) where the 5HTR1A promoter region was identified by the Ensembl data bank. A 1021 bp region of the 5HTR1A promoter

region was selected that was rich in CpG islands including 70 CG sites. The user interface of the software is shown if Figure 6.1. The target sequence was uploaded into the start-up window and Automatic judgment was selected as the primer set (parameter design condition).

Use of the "automatic judgment" selection, allows automatic calculation of the GC content from the target sequence. From this data, the sequence is then classified as one of the three sequence-types: AT rich sequence (GC% <45), normal sequence (45 < GC %< 60), or GC rich sequence (GC %> 60). The design conditions (i.e. Tm, length, and GC content) are then optimized for the target-sequence based on the sequence-type. Therefore, no additional values were entered.

Initiation of the software provided 4 sets of primers likely to have high amplification efficiency. The primers sets were identified with ID number along with changes in free energy, Tm, positions on target-sequence (i.e. 5' and 3' regions), and GC content (rate) for each individual primer and its complementary sequence that it anneals to. Gene replication by the primers occurs at the 3 prime end at the F2 region, 5 prime end at the F1c region, 3 prime end at the B2 region, and the 5 prime end at the B1c region. Because these regions are the starting point for replication, their end-stability is extremely important.

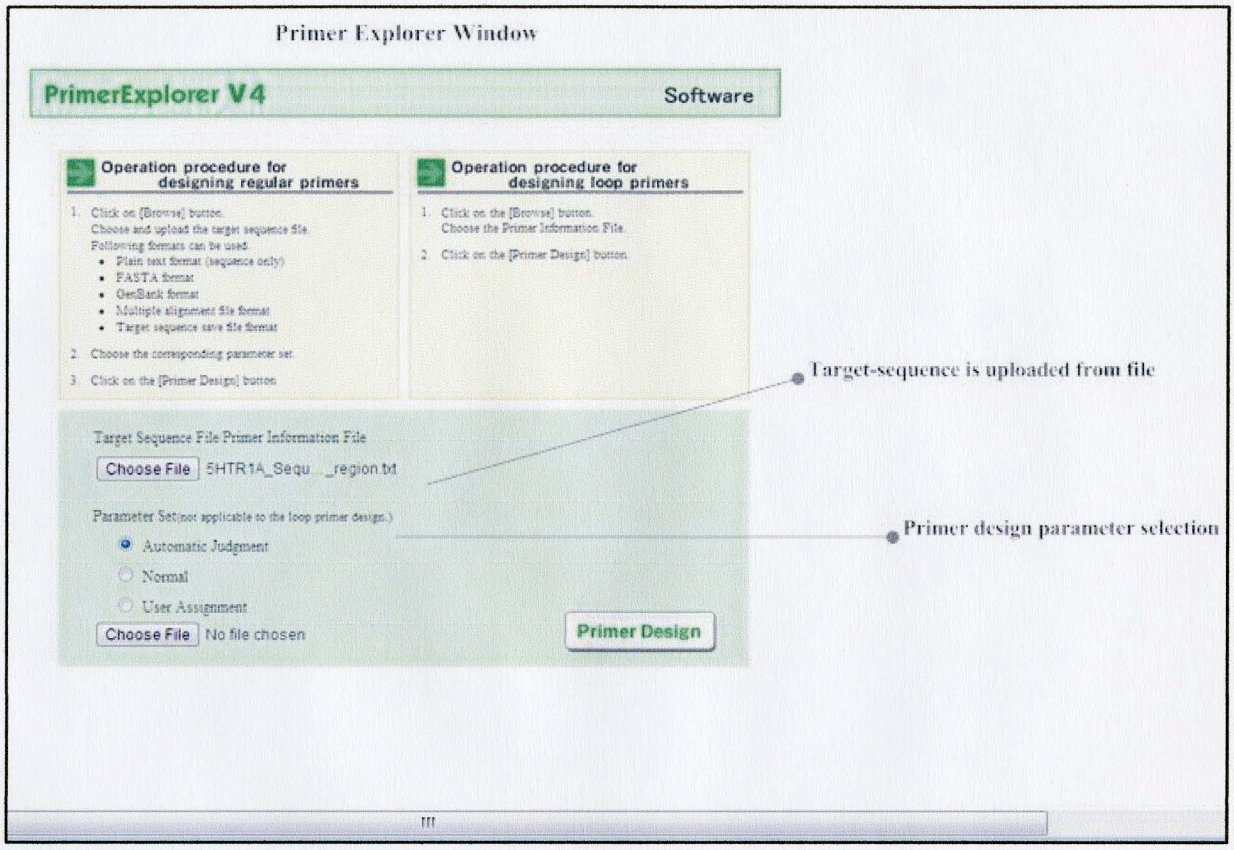

Figure 6.1 – Snapshot of the PrimerExplorer ver4 software. The target-sequence is uploaded from a text-file. Automatic Judgment is selected as the primer design parameter.

An exergonic reaction is a chemical reaction where the change in the Gibbs free energy (ΔG) is negative. The primer set chosen based on the best ΔG value of the primers at the 3 prime and 5 prime ends. ΔG indicates the tendency for dimer formation to occur. Therefore, a low ΔG (closer to zero) results in an increased chance of primer-dimer formation and thus was deemed as unacceptable. For example the end with ΔG = -5.0 kcal/mol is more stable than the end with ΔG = -4.0 kcal/mol. A good standard is to choose primers with a ΔG (stability) of -4.0 kcal/mol or lower.

The primer set selected is shown in the table below:

LAMP PRIMERS							**ID:**4	**dimer(minimum)** ΔG=-2.12
label	5'pos	3'pos	length	Tm	5'ΔG	3'ΔG	GC-rate	Sequence
F3	578	595	18	60.39	**-5.69**	**-6.41**	0.61	**GGAGGTGATCGAGGTGCA**
B3	754	773	20	59.42	**-3.92**	**-4.32**	0.50	**GATGAAGGTGCCCATGATGA**
FIP			39					**CACAAGGGGTAGGACCAGCC-GAGTGGGCAACTCCAAAGA**
BIP			40					**AGGAAAAATGAGCGCAACGCC-CCCAGCGTCTTCACTGTCT**
F2	598	616	19	59.01	**-5.35**	**-4.02**	0.53	**GAGTGGGCAACTCCAAAGA**
F1c	638	657	20	64.08	**-4.66**	**-6.69**	0.65	**CACAAGGGGTAGGACCAGCC**
B2	733	751	19	60.98	**-6.69**	**-4.74**	0.58	**CCCAGCGTCTTCACTGTCT**
B1c	675	695	21	64.20	**-4.36**	**-6.68**	0.52	**AGGAAAAATGAGCGCAACGCC**

Table 6.1 – Primer set for amplification of high GC region of 5HTR1A promoter region. Each individual primer is given with 5 prime and 3 prime ΔG values that were all over -4.00 design standards.

From the table, there are no primers with a ΔG valve of 4.00 and lower. The primers have the highest ΔG offset from zero when compared to other primer sets generated for same target-sequence. Tm for GC rich regions should be around 60–65◦C. All primers in the table are within this region with a marginal difference of 1 ◦C for B3 and F2.

Loop primers can be used to increase replication-rate during amplification (Nagamine et al. 2002). Loop primers are designed using the previously chosen standard primers. The primers are then generated based on the selected primer-set.

The loop-primers are provided in the table below:

1 ID:7 dimer(minimum)								**ΔG=-2.95**
label	5'pos	3'pos	length	Tm	5'ΔG	3'ΔG	GCrate	Sequence
LF	617	633	17	61.6	**-6.76**	**-5.40**	0.65	**TGGGCAGAGGCAAGTGC**
LB	700	719	20	65.0	**-6.03**	**-6.69**	0.65	**CGAAGCGCAAGATGGCCCTG**

Table 6.2 – Loop primers for 5HTR1A gene promoter region. Primers are generated from previous primer set.

For the loop-primers, Tm is between 65 60–65◦C with ΔG no less than 4.00.

All of the primers can be ordered from the generated data-set from a variety of vendors.

Further experimentation is then necessary to see if they can produce efficient amplification

APPENDIX A

BILL OF MATERIALS

MATERIALS	QUANTITY	ASSEMBLY	COST PER UNIT
Machined polycarbonate sheet.	1	Main-body	247.50
01" Thk, clear, laser cut polycarbonate films.	2	Top and bottom films	46.00
Acetonitrile, anhydrous, 99.8%.	10 ml	Bonding solvent	8.00
Polyimide-based thin-film heater 1"X 1"	1	Heating unit	35.50
5 % by volume Expancel 031 DU 40 microspheres	2.5 grams	Expandable microspheres	.50
FTA membrane	1	Nucleic acid isolation	5.00
Sylgard 184 Silicone Kit, Dow Corning.	50 grams	PDMS base/curing agent	6.00
LAMP Master Mix (Eiken, Teramecs).	1 reaction	LAMP reagents	4.00
STYO 9 Green Dye	1	Fluorescent dye	212.00

ESE optical dector	1	Fluorescence detection system	500.00
TOTAL			**1056.55**

APPENDIX B

Selected region of 5HTR1A promoter gene used

TGCTCAACAAGTGGACACTGGGCCAGGTAACCTGCGACCTGTTCATCGCCCT
CGACGTGCTGTGCTGCACCTCATCCATCTTGCACCTGTGCGCCATCGCGCTGG
ACAGGTACTGGGCCATCACGGACCCCATCGACTACGTGAACAAGAGGACGCC
CCGGCGCGCCGCTGCGCTCATCTCGCTCACTTGGCTTATTGGCTTCCTCATCT
CTATCCCGCCCATGCTGGGCTGGCGCACCCCGGAAGACCGCTCGGACCCCGA
CGCATGCACCATTAGCAAGGATCATGGCTACACTATCTATTCCACCTTTGGAG
CTTTCTACATCCCGCTGCTGCTCATGCTGGTTCTCTATGGGCGCATATTCCGA
GCTGCGCGCTTCCGCATCCGCAAGACGGTCAAAAAGGTGGAGAAGACCGGA
GCGGACACCCGCCATGGAGCATCTCCCGCCCCGCAGCCCAAGAAGAGTGTGA
ATGGAGAGTCGGGGAGCAGGAACTGGAGGCTGGGCGTGGAGAGCAAGGCTG
GGGGTGCTCTGTGCGCCAATGGCGCGGTGAGGCAAGGTGACGATGGCGCCGC
CCTGGAGGTGATCGAGGTGCACCGAGTGGGCAACTCCAAAGAGCACTTGCCT
CTGCCCAGCGAGGCTGGTCCTACCCCTTGTGCCCCCGCCTCTTTCGAGAGGAA
AAATGAGCGCAACGCCGAGGCGAAGCGCAAGATGGCCCTGGCCCGAGAGAG
GAAGACAGTGAAGACGCTGGGCATCATCATGGGCACCTTCATCCTCTGCTGG
CTGCCCTTCTTCATCGTGGCTCTTGTTCTGCCCTTCTGCGAGAGCAGCTGCCA
CATGCCCACCCTGTTGGGCGCCATAATCAATTGGCTGGGCTACTCCAACTCTC
TGCTTAACCCCGTCATTTACGCATACTTCAACAAGGACTTTCAAAACGCGTTT
AAGAAGATCATTAAGTGTAAGTTCTGCCGCCAGTGATGACGGAGGAGTAGCC
GGCCAGTCGAGGCTACAGGATCCGTCC

REFERENCES

Benson, D. A., M. Boguski, D. J. Lipman and J. Ostell (1996). "GenBank." Nucleic acids research 24(1): 1-5.

Birney, E. and T. Ensembl (2003). "Ensembl: a genome infrastructure." Cold Spring Harbor symposia on quantitative biology 68: 213-215.

Caragher, T. E., B. B. Fernandez, F. L. Jacobs and L. A. Barr (2002). "Evaluation of quantitative cardiac biomarker point-of-care testing in the emergency department." The Journal of Emergency Medicine 22(1): 1-7.

Carrard, A., A. Salzmann, A. Malafosse and F. Karege (2011). "Increased DNA methylation status of the serotonin receptor 5HTR1A gene promoter in schizophrenia and bipolar disorder." J Affect Disord 132(3): 450-453.

Chen, G., F. Svec and D. R. Knapp (2008). "Light-actuated high pressure-resisting microvalve for on-chip flow control based on thermo-responsive nanostructured polymer." Lab on a Chip 8(7): 1198-1204.

Chen, Z., Y. Liao, X. Ke, J. Zhou, Y. Chen, L. Gao, Q. Chen and S. Yu (2011). "Comparison of reverse transcription loop-mediated isothermal amplification, conventional PCR and real-time PCR assays for Japanese encephalitis virus." Molecular Biology Reports 38(6): 4063-4070.

Cheng, S., C. Fockler, W. M. Barnes and R. Higuchi (1994). "Effective Amplification of Long Targets from Cloned Inserts and Human Genomic DNA." Proceedings of the National Academy of Sciences of the United States of America 91(12): 5695-5699.

Cheung, K., S. Gawad and P. Renaud (2005). "Impedance spectroscopy flow cytometry:

on-chip label-free cell differentiation." Cytometry. Part A : the journal of the International Society for Analytical Cytology 65(2): 124-132.

Covault, J., C. Abreu, H. Kranzler and C. Oncken (2003). "Drug Discovery and Genomic Technologies - Quantitative real-time PCR for gene dosage determinations in microdeletion genotypes." Biotechniques. 35(3): 594.

De Luca, V., E. Viggiano, R. Dhoot, J. L. Kennedy and A. H. Wong (2009). "Methylation and QTDT analysis of the 5-HT2A receptor 102C allele: analysis of suicidality in major psychosis." J Psychiatr Res 43(5): 532-537.

Demertzis, M. A. (1988). "Fluorimetric determination of calcium in serum with calcein." Analytica Chimica Acta Analytica Chimica Acta 209: 303-308.

Donovan, B. J., J. C. Rublein, P. A. Leone and C. D. Pilcher (2004). "HIV infection: point-of-care testing." Ann Pharmacother 38(4): 670-676.

Easley, C. J., J. M. Karlinsey, J. M. Bienvenue, L. A. Legendre, M. G. Roper, S. H. Feldman, M. A. Hughes, E. L. Hewlett, T. J. Merkel, J. P. Ferrance and J. P. Landers (2006). "A fully integrated microfluidic genetic analysis system with sample-in-answer-out capability." Proc Natl Acad Sci U S A 103(51): 19272-19277.

Fang, X., H. Chen, L. Xu, J. Kong, X. Jiang and W. Wu (2012). "A portable and integrated nucleic acid amplification microfluidic chip for identifying bacteria." Lab Chip Miniaturisation Chem. Biol. Lab on a Chip - Miniaturisation for Chemistry and Biology 12(8): 1495-1499.

Fang, X., J. Kong, X. Jiang and Y. Liu (2010). "Loop-mediated isothermal amplification integrated on microfluidic chips for point-of-care quantitative detection of pathogens."

Anal. Chem. Analytical Chemistry 82(7): 3002-3006.

Freeman, W. M., S. J. Walker and K. E. Vrana (1999). "Quantitative RT-PCR: pitfalls and potential." BioTechniques 26(1): 112-122.

Galambos, P., J. Lantz, M. S. Baker, J. McClain, G. R. Bogart and R. J. Simonson (2011). "Active MEMS valves for flow control in a high-pressure micro-gas-analyzer." J Microelectromech Syst Journal of Microelectromechanical Systems 20(5): 1150-1162.

Gasparini, P., A. Grifa, P. Origone, D. Coviello, R. Antonacci and M. Rocchi (1993). "Detection of a neurofibromatosis type I (NF1) homologous sequence by PCR: implications for the diagnosis and screening of genetic diseases." Molecular and Cellular Probes 7(5): 415-418.

Gervais, L., N. de Rooij and E. Delamarche (2011). "Microfluidic chips for point-of-care immunodiagnostics." Advanced materials (Deerfield Beach, Fla.) 23(24): 151-176.
Gevertz, J. L., S. M. Dunn and C. M. Roth (2005). "Mathematical model of real-time PCR kinetics." Biotechnology and bioengineering 92(3): 346-355

Goto, M., E. Honda, A. Ogura, A. Nomoto and K. Hanaki (2009). "Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue." Biotechniques 46(3): 167-172.

Goto, M., A. Ogura, K. I. Hanaki, E. Honda and A. Nomoto (2009). "Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue." BioTechniques BioTechniques 46(3): 167-172.

Gravesen, P., J. Branebjerg and O. S. Jensen (1993). "Microfluidics-a review." Journal of Micromechanics and Microengineering 3(4): 168-182.

Griss, P., H. Andersson and G. Stemme (2002). "Expandable microspheres for the handling of liquids." Lab on a Chip 2(2): 117-120.

Han, X., L. Capretto, H. Morgan, C. Van Berkel and J. Gwyer (2012). "Microfluidic lysis of human blood for leukocyte analysis using single cell impedance cytometry." Anal. Chem. Analytical Chemistry 84(2): 1070-1075.

Heid, C. A., J. Stevens, K. J. Livak and P. M. Williams (1996). "Real time quantitative PCR." Genome research 6(10): 986-994.

Higashimoto, Y., S. Inoue, M. Ihira, A. Ohta, Y. Asano, T. Yoshikawa and C. Usui (2008). "Discriminating between varicella-zoster virus vaccine and wild-type strains by loop-mediated isothermal amplification." J. Clin. Microbiol. Journal of Clinical Microbiology 46(8): 2665-2670.

Hoelzlwallach, D. F. and M. Perezesandi (1964). "Fluorescence techniques in the microdetermination of metals in biological materials. Iii. Method for complexometric determination of magnesium in small serum samples." Analytical biochemistry 7: 67-73.

Hotzel, H., K. Sachse and H. Pfutzner (1996). "A PCR scheme for differentiation of organisms belonging to the Mycoplasma mycoides cluster." Veterinary microbiology 49(1-2): 1-2.

Iwamoto, T., T. Sonobe and K. Hayashi (2003). "Loop-mediated isothermal amplification for direct detection of Mycobacterium tuberculosis complex, M. avium, and M. intracellulare in sputum samples." Journal of clinical microbiology 41(6): 2616-2622.

Jones, P. A. and D. Takai (2001). "The role of DNA methylation in mammalian

epigenetics." Science 293(5532): 1068-1070.

Josephson, K. L., C. P. Gerba and I. L. Pepper (1993). "Polymerase chain reaction detection of nonviable bacterial pathogens." Applied and environmental microbiology 59(10): 3513-3515.

Jung, R., K. Soondrum and M. Neumaier (2000). "Quantitative PCR." Clinical chemistry and laboratory medicine : CCLM / FESCC 38(9): 833-836.

Kailasa, S. K. and S. H. Kang (2009). "Microchip-Based Capillary Electrophoresis for DNA Analysis in Modern Biotechnology: A Review." Separation & Purification Reviews 38(3): 242-288.

Kim, J., M. Mauk, D. Chen, X. Qiu, H. H. Bau, J. Kim and B. Gale (2010). "A PCR reactor with an integrated alumina membrane for nucleic acid isolation." Analyst Analyst 135(9): 2408-2414.

Kirby, B. (2010). "Micro- and nanoscale fluid mechanics transport in microfluidic devices." from http://public.eblib.com/EBLPublic/PublicView.do?ptiID=581064.
Kochanowski, B. and U. Reischl. (1999). "Quantitative PCR protocols." from http://site.ebrary.com/id/10181351.

Kryndushkin, D. S., I. M. Alexandrov, M. D. Ter-Avanesyan and V. V. Kushnirov (2003). "Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104." The Journal of biological chemistry 278(49): 49636-49643.

Kuratomi, G., K. Iwamoto, M. Bundo, T. Kato, N. Kato, I. Kusumi, N. Iwata and N. Ozaki (2008). "Aberrant DNA methylation associated with bipolar disorder identified from discordant monozygotic twins." Mol. Psychiatry Molecular Psychiatry 13(4): 429-

441.

Lagally, E. T., C. A. Emrich and R. A. Mathies (2001). "Fully integrated PCR-capillary electrophoresis microsystem for DNA analysis." Lab on a Chip 1(2): 102-107.

Lee, J.-G., K. H. Cheong, N. Huh, S. Kim, J.-W. Choi and C. Ko (2006). "Microchip-based one step DNA extraction and real-time PCR in one chamber for rapid pathogen identification." Lab on a Chip 6(7): 886-895.

Lesch, K. P. (1998). "Hallucinations: psychopathology meets functional genomics." Molecular psychiatry 3(4): 278-281.

Lesch, K. P. and L. Gutknecht (1999). "Focus on The 5-HT1A receptor: emerging role of a gene regulatory variant in psychopathology and pharmacogenetics." Int. J. Neuropsychopharm. The International Journal of Neuropsychopharmacology 7(4): 381-385.

Liu, C., E. Geva, M. Mauk, X. Qiu, W. R. Abrams, D. Malamud, K. Curtis, S. M. Owen and H. H. Bau (2011). "An isothermal amplification reactor with an integrated isolation membrane for point-of-care detection of infectious diseases." Analyst 136(10): 2069-2076.

Liu, C., M. G. Mauk and H. H. Bau (2011). "A disposable, integrated loop-mediated isothermal amplification cassette with thermally actuated valves." Microfluidics and Nanofluidics 11(2): 209-220.

Liu, P., X. Li, S. A. Greenspoon, J. R. Scherer and R. A. Mathies (2011). "Integrated DNA purification, PCR, sample cleanup, and capillary electrophoresis microchip for forensic human identification." Lab on a Chip 11(6): 1041-1048.

Louie, R. F., T. Kitano, T. K. Brock, R. W. Derlet and G. J. Kost (2009). "Point-of-care testing for pandemic influenza and biothreats." Disaster medicine and public health preparedness.

Lutz, S., P. Weber, D. Mark, R. Zengerle, F. Von Stetten, M. Focke, B. Faltin, J. Hoffmann, G. Roth, C. Muller, P. Munday, N. Armes and O. Piepenburg (2010). "Microfluidic lab-on-a-foil for nucleic acid analysis based on isothermal recombinase polymerase amplification (RPA)." Lab Chip Miniaturisation Chem. Biol. Lab on a Chip - Miniaturisation for Chemistry and Biology 10(7): 887-893.

Mark, J. E. (1999). Polymer data handbook. New York, Oxford University Press.

Matsui, K., N. Ishii, M. Honjo and Z. Kawabata (2004). "Use of the SYBR Green I fluorescent dye and a centrifugal filter device for rapid determination of dissolved DNA concentration in fresh water." AQUATIC MICROBIAL ECOLOGY. 36: 99-105.

McClelland, M. and J. Welsh (1994). "DNA fingerprinting by arbitrarily primed PCR." PCR methods and applications 4(1): 59-65.

Mills, M., P. B. Arimondo, L. Lacroix, T. Garestier, C. Hélène, H. Klump and J. L. Mergny (1999). "Energetics of strand-displacement reactions in triple helices: a spectroscopic study." Journal of molecular biology 291(5): 1035-1054.

Mocellin, S., C. R. Rossi and F. M. Marincola (2003). "Quantitative real-time PCR in cancer research." Archivum immunologiae et therapiae experimentalis 51(5): 301-313.

Monis, P. T., S. Giglio and C. P. Saint (2005). "Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye

concentration on amplification and DNA melting curve analysis." Analytical biochemistry 340(1): 24-34.

Mori, Y., K. Nagamine, N. Tomita and T. Notomi (2001). "Detection of Loop-Mediated Isothermal Amplification Reaction by Turbidity Derived from Magnesium Pyrophosphate Formation." Biochemical and Biophysical Research Communications 289(1): 150-154.

Mori, Y. and T. Notomi (2009). "Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases." Journal of Infection and Chemotherapy 15(2): 62-69.

Nagamine, K., T. Hase and T. Notomi (2002). "Accelerated reaction by loop-mediated isothermal amplification using loop primers." Molecular and Cellular Probes 16(3): 223-229.

Nairn, C., A. Lovatt and D. N. Galbraith (2003). "Detection of infectious bovine polyomavirus." Biologicals : journal of the International Association of Biological Standardization 31(4): 303-306.

Njiru, Z. K., A. S. Mikosza, T. Armstrong, J. C. Enyaru, J. M. Ndung'u and A. R. Thompson (2008). "Loop-mediated isothermal amplification (LAMP) method for rapid detection of Trypanosoma brucei rhodesiense." PLoS neglected tropical diseases 2(1).

Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino and T. Hase (2000). "Loop-mediated isothermal amplification of DNA." Nucleic Acids Research 28(12): e63.

Oh, K. W. and C. H. Ahn (2006). "A review of microvalves." Journal of micromechanics

and microengineering : structures, devices, and systems. 16(5): R13-R39.

Ohori, A., S. Endo, A. Sano, K. Yokoyama, K. Yarita, M. Yamaguchi, K. Kamei, M. Miyaji and K. Nishimura (2006). "Rapid identification of Ochroconis gallopava by a loop-mediated isothermal amplification (LAMP) method." Veterinary Microbiology 114(3-4): 359-365.

Olabisi, O. (1997). "Handbook of thermoplastics." from http://search.ebscohost.com/login.aspx?direct=true&scope=site&db=nlebk&db=nlabk&AN=12621.

Olasagasti, F. and J. C. Ruiz de Gordoa (2012). "Miniaturized technology for protein and nucleic acid point-of-care testing." Translational Research 160(5): 332-345.

Pamme, N. (2005). "Magnetism and microfluidics." Lab on a Chip 6(1): 24-38.

Parida, M., K. Horioke, H. Ishida, P. K. Dash, P. Saxena, A. M. Jana, M. A. Islam, S. Inoue, N. Hosaka and K. Morita (2005). "Rapid detection and differentiation of dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay." Journal of clinical microbiology 43(6): 2895-2903.

Parida, M., G. Posadas, S. Inoue, F. Hasebe and K. Morita (2004). "Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus." Journal of clinical microbiology 42(1): 257-263.

Price, C. P. (2003). "Point-of-Care Testing in Diabetes Mellitus." Clinical Chemistry & Laboratory Medicine 41(9): 1213-1219.

Ronaghi, M. (2001). "Pyrosequencing sheds light on DNA sequencing." Genome

research 11(1): 3-11.

Roxhed, N., S. Rydholm, B. Samel, W. van der Wijngaart, P. Griss and G. Stemme (2006). "A compact, low-cost microliter-range liquid dispenser based on expandable microspheres." Journal of micromechanics and microengineering : structures, devices, and systems. 16(12): 2740-2746.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich (1988). "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase." Science (New York, N.Y.) 239(4839): 487-491.

Samel, B., P. Griss and G. Stemme (2007). "A Thermally Responsive PDMS Composite and Its Microfluidic Applications." JOURNAL OF MICROELECTROMECHANICAL SYSTEMS 16(1): 50-57.

Samel, B., V. Nock, A. Russom, P. Griss and G. Stemme (2007). "A disposable lab-on-a-chip platform with embedded fluid actuators for active nanoliter liquid handling." Biomedical Microdevices 9(1): 61-67.

Schmittgen, T., B. Zakrajsek, A. Mills, V. Gorn, M. Singer and M. Reed (2000). "Quantitative Reverse Transcription-Polymerase Chain Reaction to Study mRNA Decay: Comparison of Endpoint and Real-Time Methods." Analytical Biochemistry 285(2): 194-204.

Shampo, M. A. and R. A. Kyle (2002). "Kary B. Mullis--Nobel Laureate for procedure to replicate DNA." Mayo Clinic proceedings. Mayo Clinic 77(7).

Shao, P., Z. Rummler and W. K. Schomburg (2004). "Polymer micro piezo valve with a small dead volume." Journal of micromechanics and microengineering : structures,

devices, and systems. 14: 305-309.

Sharma, S. D. and K. Sagara (2005). "Latent Heat Storage Materials and Systems: A Review." International Journal of Green Energy 2(1): 1-56.

Soliman, H. and M. El-Matbouli (2005). "An inexpensive and rapid diagnostic method of Koi Herpesvirus (KHV) infection by loop-mediated isothermal amplification." Virology journal 2.

Taberlet, P., S. Griffin, B. Goossens, S. Questiau, V. Manceau, N. Escaravage, L. P. Waits and J. Bouvet (1996). "Reliable genotyping of samples with very low DNA quantities using PCR." Nucleic acids research 24(16): 3189-3194.

Tao, Z. Y., H. Y. Zhou, H. Xia, S. Xu, H. W. Zhu, R. L. Culleton, E. T. Han, F. Lu, Q. Fang, Y. P. Gu, Y. B. Liu, G. D. Zhu, W. M. Wang, J. L. Li, J. Cao and Q. Gao (2011). "Adaptation of a visualized loop-mediated isothermal amplification technique for field detection of Plasmodium vivax infection." Parasit Vectors 4: 115.

Thuillier, G. and C. Malek (2005). "Development of a low cost hybrid Si/PDMS multi-layered pneumatic microvalve." Microsystem Technologies 12(1-2): 1-2.

Tomita, N., Y. Mori, H. Kanda and T. Notomi (2008). "Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products." Nat. Protoc. Nature Protocols 3(5): 877-882.

Tourlousse, D. M., F. Ahmad, R. D. Stedtfeld, G. Seyrig, S. A. Hashsham and J. M. Tiedje (2012). "A polymer microfluidic chip for quantitative detection of multiple water- and foodborne pathogens using real-time fluorogenic loop-mediated isothermal amplification." Biomed. Microdevices Biomedical Microdevices 14(4): 769-778.

Tsujita, T., N. Niikawa, H. Yamashita, A. Imamura, A. Hamada, Y. Nakane and Y. Okazaki (1998). "Genomic discordance between monozygotic twins discordant for schizophrenia." The American journal of psychiatry 155(3): 422-424.

VanGuilder, H. D., K. E. Vrana and W. M. Freeman (2008). "Twenty-five years of quantitative PCR for gene expression analysis." BioTechniques BioTechniques 44(5): 619-626.

Wang, C. H. and G. B. Lee (2005). "Automatic bio-sampling chips integrated with micro-pumps and micro-valves for disease detection." Biosensors & bioelectronics. 21(3): 419-425.

Wang, D., M. Szyf, C. Benkelfat, N. Provencal, G. Turecki, D. Caramaschi, S. M. Cote, F. Vitaro, R. E. Tremblay and L. Booij (2012). "Peripheral SLC6A4 DNA methylation is associated with in vivo measures of human brain serotonin synthesis and childhood physical aggression." PLoS One 7(6): e39501.

Wang, J., Z. Chen, M. Mauk, K.-S. Hong, M. Li, S. Yang and H. Bau (2005). "Self-Actuated, Thermo-Responsive Hydrogel Valves for Lab on a Chip." Biomedical Microdevices 7(4): 313-322.

Wang, J., Z. Chen, M. Mauk, K. S. Hong, M. Li, S. Yang and H. H. Bau (2005). "Self-actuated, thermo-responsive hydrogel valves for lab on a chip." Biomed Microdevices 7(4): 313-322.

Wei, X., X. Shi, Y. Zhao, J. Zhang, M. Wang, C. Liu, H. Cui, S. Hu, Y. Quan, H. Chen and Y. Wang (2012). "Development of a rapid and specific loop-mediated isothermal amplification detection method that targets Marek's disease virus meq gene." Journal of

Virological Methods 183(2): 196-200.

Whelan, J. (2001). "Microscale valves drive progress in microfluidics." Drug discovery today 6(22): 1134-1135.

Whitcombe, D., J. Theaker, S. P. Guy, T. Brown and S. Little (1999). "Detection of PCR products using self-probing amplicons and fluorescence." Nature biotechnology 17(8): 804-807.

Yager, P., T. Edwards, E. Fu, K. Helton, K. Nelson, M. R. Tam and B. H. Weigl (2006). "Microfluidic diagnostic technologies for global public health." Nature 442(7101): 412-418.

Yokoyama, E., M. Uchimura and K. Ito (2010). "Detection of enteroaggregative Escherichia coli by loop-mediated isothermal amplification." Journal of food protection 73(6): 1064-1072.

You, D. J., T. S. Park, J. Y. Yoon and B. Selected Papers from the World Congress on (2013). "Cell-phone-based measurement of TSH using Mie scatter optimized lateral flow assays." Biosensors and Bioelectronics 40(1): 180-185.

Zhang, C. and D. Xing (2010). "Decreasing microfluidic evaporation loss using the HMDL method: Open systems for nucleic acid amplification and analysis." Microfluid. Nanofluid. Microfluidics and Nanofluidics 9(1): 17-30.

Zipper, H., H. Brunner, J. Bernhagen and F. Vitzthum (2004). "Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications." Nucleic Acids Research 32(12).